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1) Publications from the Department of Agricultural and Biological Chemistry, the Pennsylvania State College, 1923-1930, no. 5, 7, 10, 12-17, 20, 22, 24, 27-70
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2) Publications from the Department of Agricultural and Biological Chemistry, the Pennsylvania State College, 1931-1935, no. 71-73, 75-91, 93-110, 112-117,119-129, 132-134
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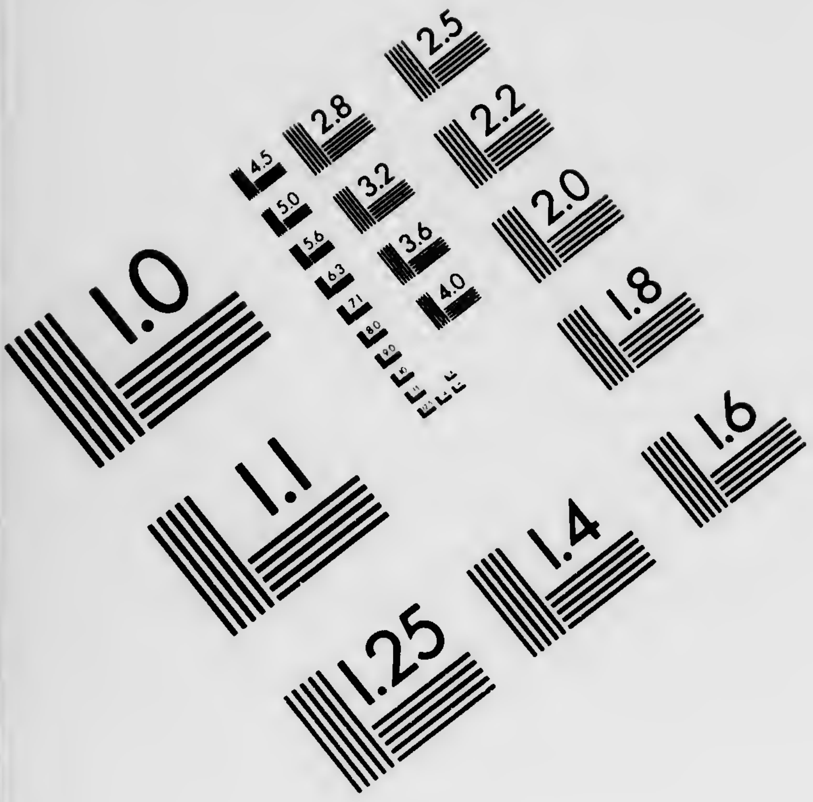

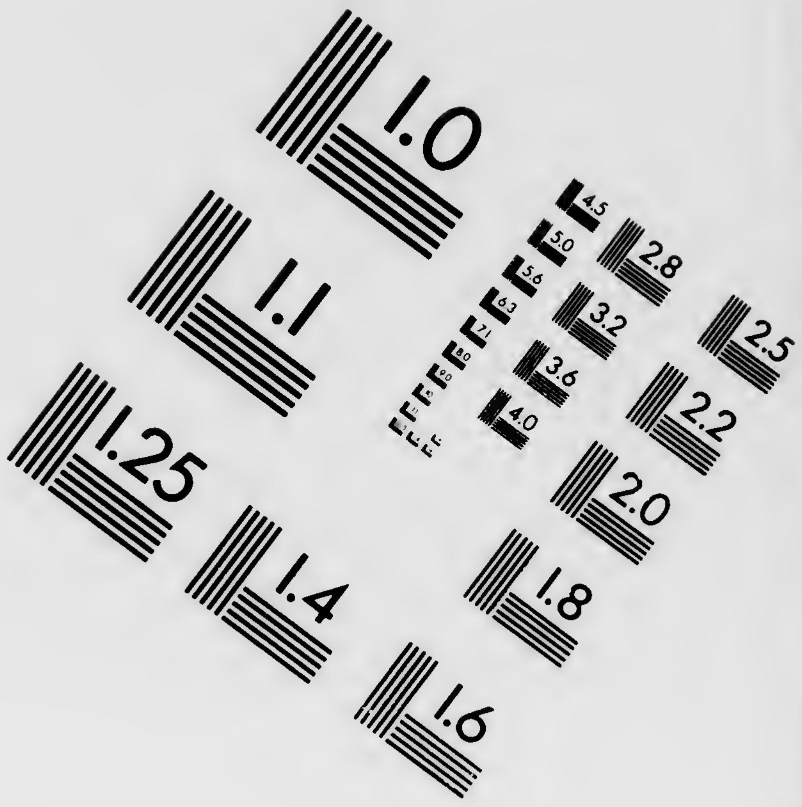

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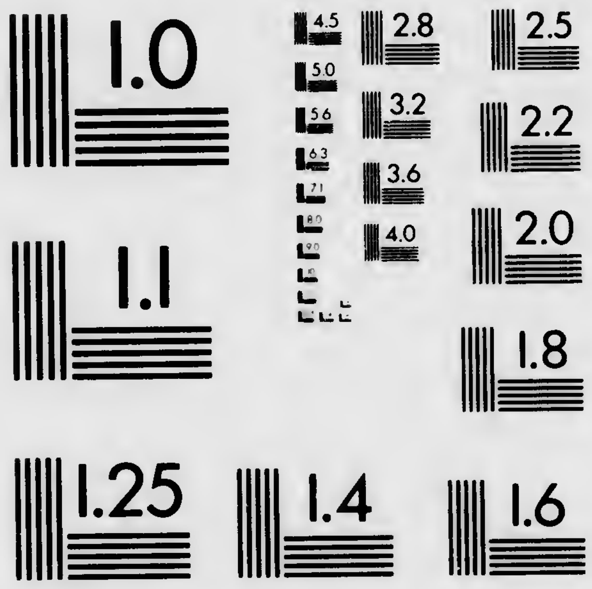

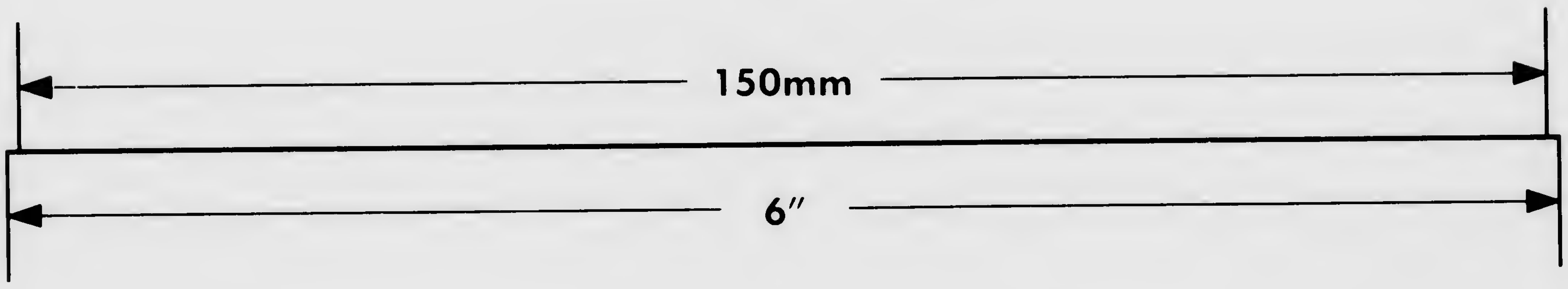

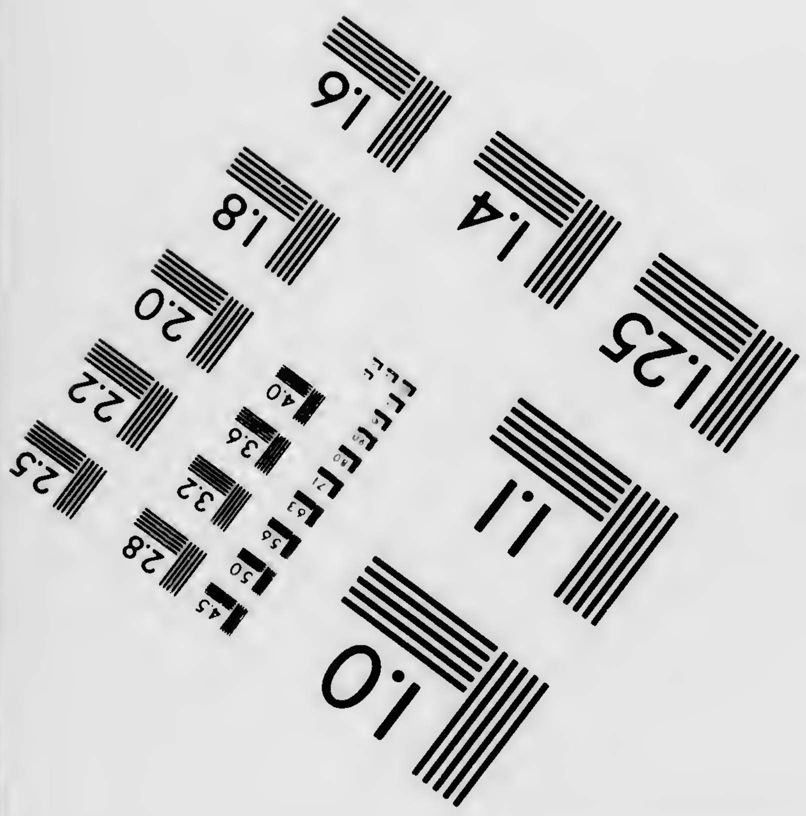

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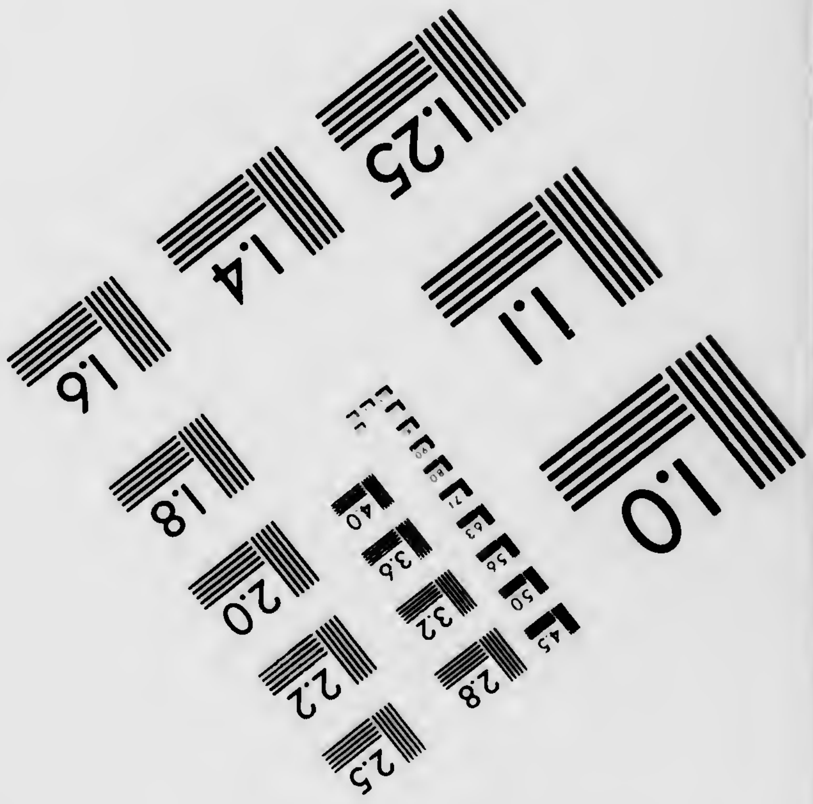

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ULTIMATE ANALYSIS OF THE MINERAL CONSTITUENTS OF A HAGERSTOWN SILTY CLAY LOAM SOIL AND OCCURRENCE IN PLANTS OF SOME OF THE ELEMENTS FOUND[1]

WALTER THOMAS[2]
Pennsylvania Agricultural Experiment Station.

Received for publication July 1, 1922

INTRODUCTORY

The soil for which analysis is here reported is being used in a 20-year cooperative experiment between the Departments of Horticulture and of Experimental Agricultural Chemistry. This experiment is being conducted in steel rims, forty-two in number, to determine the effect of fertilizers on the yield, growth, and other physiological functions of the apple tree. It is the result of inconclusive chemical studies conducted during 1917–19 on the soils and plants of the experimental orchards throughout the state. The variables have been eliminated in the present investigation as nearly as possible by using a uniform soil obtained by thorough and complete mixing of the sub-, subsurface, and surface-soil and also by means of a uniform stock. The soil with which these rims were filled was taken from a strip of virgin land adjacent to the experimental orchard, which lies about a mile and a half to the northeast of the well known general fertilizer plats, at the junction of the Stonehenge and Trenton formations, and is classed by Mooney, Shaw et al. (55), as a clay loam of the Hagerstown series. However, Shaw recognizes considerable variations in the texture and other physical qualities of these soils, e.g. he distinguished as present in the general fertilizer plats the following sub-classes: silty clay loam, heavy loam, clay loam and clay. The mechanical analyses (cf. table 1) of the soil in this experiment would indicate that it is a mixture of the Hagerstown silt loam and clay loam. This soil is probably formed by the disintegration of limestone rocks and represents that portion of the soil mass adjacent to the parent rock.

In an intensive experiment of this character it was regarded as fundamental to have as complete a knowledge not only of the total constituents of the soil but also of the total amount of each element present, which can only be obtained by making a complete examination following the fusion method for rock analyses and adopting quantitative procedures of approved validity.

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[1] Contribution No. 1 from the laboratories of the Department of Agricultural Chemistry of the Pennsylvania State College Agricultural Experiment Station.

[2] The mineralogical section of this paper (cf. p. 18) is based on the micro-petrographical examination made by Professor A. P. Honess. The author wishes to take this opportunity to express his indebtedness and gratitude for the guidance given him by the late Dr. William Frear with whom the author has been associated for nearly 10 years and under whose general direction the chemical phase of this project was initiated.

Robinson (62, p. 12–13) has reported the occurrence of a number of the rarer elements in a Hagerstown loam soil taken one mile northwest of Conshohocken, Pa., which is not far from metamorphic rocks. Fragments of mica schist (1.8 per cent of the stony fragments) occur in the surface soil. This showing is quite different from the conditions at State College, where the soil has been formed in place by weathering of the limestone belonging to the lower Silurian formations. The existence of these elements in this soil is conceivably susceptible of several explanations. If strictly residual then the origin would be the underlying limestone, but it is very questionable if these rarer elements would be found in the limestone underlying the soil. This point will be investigated later. As Merrill (54, p. 4–8) points out, the agencies which have been instrumental in the formation of soils have been so complex that many of our soils are but secondary rocks in a state of loose consolidation, and many of the accumulations classed as residual have been derived by disintegration in situ of alluvial materials which have been brought down years ago and deposited in shallow seas. Moreover, because of the transporting power of water, wind, or moving ice, few residual deposits have retained their virgin purity, but have become more or less contaminated with materials from near or distant sources.

Dr. E. S. Moore, Dean of the School of Mines of the Pennsylvania State College is of the opinion that these rarer elements have, probably, been derived "from the extensive pre-Cambrian area, known as the Appalachia, which existed to the east and northeast of this region at the time these Palaeozoic rocks were being laid down."

THE ISOLATION OF THE RARER ELEMENTS IN THIS RESIDUAL SOIL

Clarke (11, p. 15–22), Merrill (54, p. 312), Washington (75), and Hillebrand (42, p. 25–30) have been consulted in obtaining the following data on the soil used in this study:

Aluminum: Found chiefly in silicates such as the feldspars and the micas. It occurs in this soil in the mixed feldspars, $R'AlS_3iO_8$, in which R′ may be Ca, Mg, Na or K; mica, $H_2(KNa)Al_3(SiO_4)_3$; tourmaline, $R'_9Al_3B_2(OH)_2Si_4O_{19}$, in which R′ may be Fe, Mg or alkali metals; and chlorite, $H_8(Mg,Fe)_5Al_2(SiO_6)_3$.

Barium: Widely distributed in small quantities throughout igneous rocks. Professor Honess has found barite in sediments near Bellefonte, Pa., and has called the attention of the writer to the statement by Dana (19) that it has been proven to exist in amounts varying from 0.45 per cent to 2.20 per cent in orthoclase and some acid plagioclases, which are probably the original sources of barium, from which it is dissolved. It occurs also as carbonate and witherite. Failyer (27) reports that barium is present in most soils throughout the United States.

Boron: An essential constituent of several silicates. It is present in this soil in tourmaline, which has been found by Doctor Moore in the Tuscarora sandstones and quartzites.

Caesium: A rare metal—the rarest of the alkalies. Often found in lepidolite, $(Li,K)_2(FOH)_2Al_2SiO_3$; Vernadski (69, 70) found spectroscopic traces in the feldspars and micas, which are the probable sources of the element in this soil.

Calcium: An essential constituent of many rock-forming minerals such as the amphiboles and the pyroxenes. It occurs as dolomite, $CaMg(CO_3)_2$, and amphibole, $Ca(MgFe)_3(SiO_3)_4$. Sericite and the mixed feldspars found in this soil contain small amounts of calcium.

Chromium: Very widely diffused in the form of chromite, $(Fe,Cr)[(Cr,Fe)O_2]_2$, and especially in ferro-magnesian rocks. Its source in this soil is probably chlorite.

Fluorine: Fluorine is found in a number of silicates such as tourmaline and the micas, which are its probable source in this soil.

Iron: Found in practically all rocks. Occurs as limonite, $H_2Fe_2O_4(H_2O)_4$; ilmenite $FeTiO_3$; magnetite, Fe_3O_4; and also chlorite and amphibole in this soil.

Lithium: Traces are found in nearly all igneous rocks. The most important lithia minerals are lipidolite, $(Li,K_2)(F,OH)_2Al_2Si_3O_9$; spodumene $LiAl(SiO_3)_2$; and the lithia tourmalines and alkali feldspars. Washington (75, p. 20) states that it frequently occurs in rocks high in sodium.

Magnesium: Magnesium occurs in this soil as amphibole, dolomite and chlorite. The micas also contain magnesium.

Manganese: Found in most rocks and soils in small amounts in the ferro-magnesian minerals. Through alteration it appears sometimes on the surfaces of limestones and sandstones. Its probable source in this soil is chlorite.

Molybdenum: Hillebrand (42, p. 185) has found it in minute quantities in siliceous rocks. It has also been found in granite in the form of molybdenite, MoS_2, but its source in this soil has not been determined.

Phosphorus: Found in nearly all igneous rocks in the mineral apatite. Its source in this soil has not been determined unless it occurs in the limonite (cf. p. 11).

Potassium and sodium: These metals are present in this soil in the mixed feldspars and micas.

Rubidium: The source of this metal in this soil has not been determined. It is found in lepidolite, $(Li,K)_2(F,OH)_2Al_2Si_2O_9$, and in some mineral springs. It is reported as being present in the waters of the Caspian Sea.

Strontium: This is a common ingredient of igneous rocks in small amounts. The most important minerals are celestite, $SrSO_4$, and strontianite, $SrCo_3$. Lesley (47) reports the absence of celestite in the beds of this limestone formation. Professor Honess, however, has called to the author's attention the fact that Dana (20) states that it occurs in the limestone regions of Central Pennsylvania, at Bells Mills, Blair County. It has recently been found by Mr. A. H. Dewey of the School of Mines of this Institution at Williamsburg, Pa.

Sulfur: The source of sulfur in this soil has not been determined. It certainly exists as sulfates and in organic combination. No sulfide minerals were found.

Titanium: Titanium is almost invariably present in igneous rocks and the sedimentary material derived from them. It occurs in this soil as ilmenite, $FeTiO_3$, and rutile, Ti,TiO_4.

Vanadium: This is diffused with titanium through all primitive granite rocks, has been found in rutile and bauxite, and is reported as occurring very commonly in small amounts in residual limestone deposits, which is probably the source of the vanadium found in this soil. Phillips (58) suggests that vanadium in sedimentary rocks may be derived from the decay of mineral organisms—the acidians.

Zirconium: This is allied to titanium and rather widely diffused in the igneous rocks and has been found by Doctor Moore in the Tuscarora sandstones and quartzites. Hillebrand (42, p. 25–30) and Robinson (62, p. 3) state that it occurs in largest amounts in soils high in silica and sodium.

The present investigation is of special interest because of the isolation by quantitative methods of known validity and confirmatory qualitative tests of the rarer elements vanadium, chromium, molybdenum, zirconium, titanium, rubidium, caesium, lithium and also of the elements barium and strontium, concerning the presence of which in this soil series much doubt has existed.

THE DISTRIBUTION OF THE ELEMENTS PRESENT IN THIS SOIL

MECHANICAL ANALYSIS

The mechanical analysis was carried out by the method described by the Bureau of Soils (8, p. 7–24) on samples prepared according to the methods prescribed by the Association of Official Agricultural Chemists (2) dried at 110°C. Duplicate determinations were made and are shown in table 1.

It is of interest to note the difference in mechanical composition of this soil and that of the grass lands of the general fertilizer plats as given by Frear and White (30), in table 2.

The soil used in the apple rim experiment is, therefore, of a much more clayey texture than the major portion of the land of the college farm, though the relatively larger amount of clay present is not sufficient to interfere with the ease of tillage, excellence of drainage and formation of clods on drying, which are characteristic of this soil type.

TABLE 1

GRADE	SURFACE SOIL TO 9.5 IN.		SUBSURFACE SOIL 9.5–18.5 IN.		SUBSOIL 18.5–42 IN.	
	(a)	(b)	(a)	(b)	(a)	(b)
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Coarse sand, 1.0–0.5 mm	7.30	7.52	5.45	5.43	4.43	4.45
Medium sand, 0.5–0.25 mm	4.82	5.20	3.90	3.74	3.51	3.40
Fine sand, 0.25–0.1 mm	3.40	3.40	3.07	3.00	3.05	3.01
Very fine sand, 0.1–0.05 mm	12.80	12.51	14.00	13.98	12.22	12.50
Silt, 0.05–0.005 mm	50.30	50.52	45.43	46.00	46.45	47.44
Clay, 0.005 mm. or less	15.78	15.67	23.29	22.76	25.57	24.33
Loss on ignition	5.30	5.30	5.00	5.00	5.50	5.50
Total	99.70	100.12	100.14	99.91	100.73	100.63

TABLE 2

GRADE	SURFACE SOIL TO 7 IN.	SUBSURFACE, 7–14 IN.
	per cent	*per cent*
Very fine sand	6.44	6.78
Silt	75.05	67.83
Clay	9.45	17.98

Samples were taken from each wheelbarrow load as the rims were being filled. These were placed in cans and stored as "surface-", "subsurface-" and "sub-" soil. The samples from each of these three layers were treated as follows: The whole soil sample, about 270 kgm., was placed upon heavy paper, thoroughly mixed by turning over with a hand scoop, and divided into four segments in the usual way. The process of mixing and quartering was continued until the quantity of soil was reduced to about 2 kgm.

The three samples resulting were air-dried, washed and sieved as prescribed by the method of the Association of Official Agricultural Chemists (2). About 50 gms. of each sample, was finely ground in an agate mortar until all passed through a two hundred mesh silk bolting cloth.

THE CHEMICAL ANALYSIS

The complete chemical analysis of the soil, in general as outlined by Hillebrand (42), is given in table 3.

The analysis of the various soil fractions has not been undertaken. Failyer, Smith and Wade (28) have shown that as a general rule the smaller particles of soils are richer in potassium, calcium, magnesium and phosphorus than the coarser particles and the concentration becomes greater the greater the weathering, and that the larger mechanical fractions contain these elements in forms which by protracted weathering will become more soluble and will ultimately be concentrated in the finer compounds.

TABLE 3

CONSTITUENT	SURFACE SOIL TO 9.5 IN.		SUBSURFACE SOIL 9.5–18.5 IN.		SUBSOIL 18.5–42 IN.	
	(a)	(b)	(a)	(b)	(a)	(b)
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
SiO_2	72.320	72.340	67.720	67.620	63.230	63.000
Al_2O_3	10.192	10.322	13.503	13.667	15.994	15.974
Fe_2O_3	3.710	3.670	5.580	5.580	6.840	6.840
FeO	0.540	0.550	0.350	0.350	0.207	0.207
V_2O_5	0.036	0.036	N E	N E	0.090	0.080
Cr_2O_3	0.002	0.002	N E	N E	0.002	0.002
MoO_2	P*	P	N E	N E	P	P
TiO_2	0.340	0.320	0.480	0.460	0.440	0.480
P_2O_5	0.102	0.095	0.102	0.098	0.077	0.078
ZrO_2	0.037	(0.037)†	0.045	(0.045)	0.050	(0.050)
MnO	0.240	0.230	0.250	0.260	0.230	0.260
CaO	0.660	0.700	0.650	0.660	0.900	0.910
MgO	0.650	0.680	0.980	1.050	1.370	1.350
BaO	0.009	(0.009)	0.019	0.020	0.019	0.019
SrO	0.016	0.010	0.022	0.018	0.033	0.028
Rb	0.001	0.001	N E	N E	0.001	0.001
K_2O	3.960	3.890	4.220	4.180	4.420	4.400
Cs	N F	N F	N E	N E	P	P
Na_2O	2.280	2.240	1.280	1.300	1.350	1.280
Li	Trace	Trace	Trace	Trace	Trace	Trace
Cl	Trace	Trace	Trace	Trace	Trace	Trace
S	0.062	(0.062)	0.051	(0.051)	0.062	(0.062)
SO_3	0.017	0.017	0.020	0.020	0.017	0.017
Loss on ignition	5.300	(5.300)†	5.000	(5.000)	5.500	(5.500)
Total	100.474	100.511	100.272	100.379	100.832	100.538
Less oxygen equivalent of S	100.448	100.485	100.250	100.357	100.806	100.512

* P = present; N E = not examined; N F = not found.

† Parentheses indicate that duplicate determinations were not made.

The mineralogical examination of this soil (cf. p. 10) confirms these conclusions.

NOTES ON THE CHEMICAL ANALYSIS

1. Before mixing with sodium carbonate prior to the fusion, the charge was ignited to destroy organic matter.

2. The silica not obtained by the fusion with HCl, after three evaporations and intervening filtrations (41), was separated from the mixed oxides, of alumina, etc., by fusion with potassium bisulfate and added to the weight of the silica already obtained. The weight of silica was corrected for impurities by evaporation in HF and H_2SO_4.

3. The precipitation of iron and aluminum was made with ammonia by Blum's procedure (6). The author found no manganese, alkaline earth metals or magnesium in this precipitate.

4. Manganese was determined by the ammonium sulfide method. The amount of manganese that escaped precipitation was negligible, for none was found by the colorimetric method in the magnesium pyrophosphate precipitate in which, if any was present, it would occur.

5. The results for ferrous iron are to be regarded as approximate. The various devices used to prevent oxidation of the FeO to Fe_2O_3 are fully discussed by Hillebrand (42, p. 189–192) and Washington (75, p. 122–126). The presence of organic matter, moreover, might reduce some of the ferric iron during the operations. Cooke's method (15) simplified by Barneby (3) was adopted.

6. Nickel, cobalt, copper, zinc, mercury, silver, tin, lead and arsenic were not found.

7. Calcium and strontium were precipitated as oxalates and separated, after conversion into the nitrates, by the ether-alcohol method (32). The strontium was weighed as the sulfate, which was examined by the spectroscope.

The orange α line of wave length 6060 and the blue δ line of wave length 4607.5 identified Sr. The band spectrum of calcium with characteristic line of wave length 4226.9 was also observed. This examination confirms the presence of strontium in this soil, though complete separation from calcium was not effected. It is remarkable that, though barium was absolutely identified, no barium lines were observed in this separate, showing complete separation from this element.

8. Barium together with zirconium, total sulfur and rare earths were determined by fusion with sodium carbonate and a little potassium nitrate on a separate 2-gm. charge of the soil. The zirconium and rare earths were separated from barium by means of dilute warm sulfuric acid. After evaporating the residue remaining undissolved with hydrofluoric acid and sulfuric acid, it was freed from calcium and strontium by repeated fusions with sodium carbonate, solution of the melts in water and reprecipitation with sulfuric acid. The barium sulfate obtained in this manner was dissolved in concentrated sulfuric acid and reprecipitated by water to remove the last traces of calcium. The barium sulfate thus obtained was ignited, weighed and examined by the spectroscope.

The green barium line of wave length 5535.7 was used for identification. Separation from calcium and strontium was completed, as none of the characteristic lines of these elements were present.

9. The method which Robinson (62, p. 7; 64), used to estimate the amounts of rubidium and caesium was adopted. Ten-gram portions of soil were used. The method removes a large part of the potassium and sodium chlorides present by fractional precipitation with platinic chloride and then by strong hydrochloric acid. The platinum was removed from the double platinic chlorides Cs_2PtCl_6 and Rb_2PtCl_6 by Horsch's method (44). After filtering and evaporation, the weights of the mixed chlorides from the surface and subsoil were 0.8 and 0.2 mgm. respectively. After treatment with hydrochloric acid, it was filtered into small vials and examined by means of the spectroscope, using the flame spectrum, according to the method of Gooch and Phinney (37).

The blue rubidium lines of wave length 4215.6 and 4202 were clearly defined in both the case of the surface and subsoil. In the case of the subsoil, but not of the surface soil, a doublet of wave length 4593.3 and 4555.4 was plainly discernible.

10. The examination for lithium was made on the solution obtained after removal of the potassium as potassium platinic chloride from the mixed chlorides of potassium and sodium. The platinum was removed by Horsch's method. The lithium lines of wave length 6708.2 and 5104 were observed.

An attempt was made to effect a separation of the lithium and sodium by Gooch's method (36), but the amounts of lithium chloride obtained were too small to obtain pure.

11. Calcium was always found in the magnesium pyrophosphate and was removed by treatment of the latter with dilute sulfuric acid and alcohol. The calcium sulfate obtained was filtered, weighed, and suitable corrections made in the figures for calcium and magnesium oxides.

12. The zirconium, rare earths, and total sulfur were determined by fusing 2-gm. portions of the soil powder (see note 8).

The zirconium pyrophosphate obtained by ignition of the phosphate was freed from any titanium that might possibly be present by fusion with sodium carbonate, leaching with water, fusion of the residue with potassium pyrophosphate, solution of the melt in 20 per cent sulfuric acid [Recolardot and Reglade (61)] to prevent contamination with titanium, iron, and chromium with the addition of hydrogen peroxide and reprecipitating with sodium hydrogen phosphate. The precipitate was ignited and weighed as the pyrophosphate.

Certainty as to its identification was again established by fusing with sodium carbonate, leaching, igniting the residue, fusing with potassium pyrophosphate and reprecipitating with ammonia, filtering, igniting and weighing as ZrO_2. This was then dissolved in concentrated sulfuric acid, precipitated with ammonia, dissolved in hydrochloric acid and tested with tumeric paper after evaporation almost to dryness.

13. The rare-earth metals were sought in the filtrate from the zirconium phosphate by precipitating with KOH and treating the precipitate with HF, converting into sulfates, precipitating with ammonia, and finally converting into the oxalates. The amounts were extremely small and no concordant results could be obtained.

14. Vanadium, chromium and molybdenum were determined by the Hillebrand (42, p. 185) method on a 5-gm. charge of the soil powder.

Confirmatory qualitative tests for the presence of vanadium and molybdenum were applied as follows: The solution was evaporated to dryness and heated to expel excess of sulfuric acid. The residue was taken up with 2–3 cc. of water containing a few drops of dilute nitric acid. On addition of a few drops of hydrogen peroxide a characteristic brownish tint developed. Certainty as to the identity of molybdenum was established by igniting the sulfide in porcelain and adding a single drop of concentrated sulfuric acid. On heating, a beautiful blue color indicated the presence of molybdenum.

15. The literature on the determination of sulfur as barium sulfate is voluminous. The total sulfur was determined by fusion with sulfur-free sodium carbonate and about 0.5 gm. potassium nitrate, solution of the melt in water, reduction of the manganese with ethyl alcohol and precipitation as barium sulfate. In connection with this determination it is interesting to note that Hillerbrand has shown, contary to the experience of Stoddart (67), that it is unnecessary to remove the silica. For, if the filtrate measures about 100–200 cc. and is acidified only slightly with hydrochloric acid in the cold, no separation of the silica occurs. This is indeed fortunate for in the case of soils the amount of silica is so high that filtration from the silica would be an almost impossible task. The writer found absolutely no separation of silica by this method. The sulfur given in the tabulated statement was obtained by subtracting from the total sulfur that present as SO_3, obtained by treating with 100 cc. of $16\frac{2}{3}$ per cent HCl.

16. The loss on ignition has no particular significance. It represents the algebraic sum of a number of changes depending on the temperature employed. With increasing temperature the sulfates are decomposed, all the sulfur being driven off, at which point the alkalies begin to escape. The figures, which are the average of a large number of determinations, are to be regarded as approximations only, for it is difficult to prevent reduction by the organic

matter, even if a low temperature with proper precautions is employed. This fact together with the difficulty of dehydrating the hydrated minerals like mica, limonite and chlorite accounts for the summation being over 100.

17. Boron and fluorine exist in tourmaline and mica respectively, both of which have been found in this soil.

The author's attempts at a quantitative estimation of these elements in this soil have not met with much success. Cook (12) and Cook and Wilson (13, 14), have described without giving details the methods used in estimating boron in soils. The author, however, could not obtain consistent results with the colorimetric method used by these authors or by the method outlined by Hillebrand (42, p. 234–238). The objection found to the colorimetric method was the inability to secure reagents used in the test that gave no color with filter paper dipped in a 0.2-per cent solution of curcumin. The amount present was too small to obtain any success with Hillebrand's method.

18. Two, and sometimes three precipitations were carried out throughout this work. Wherever practicable evaporations and precipitations were made at the boiling point in platinum dishes. The filtrations were made into pyrex glass beakers. Blank determinations were made on all reagents.

DISCUSSION OF THE ANALYSIS

Examination of the results indicates that the surface and subsurface soil contain more phosphoric acid than the subsoil. Sodium is much higher in the surface soil than in the other two layers, whereas the subsoil is richest in calcium, magnesium and potassium.

The tendency of iron to work its way down is manifest, the subsoil containing almost twice as much Fe_2O_3 as the surface soil. Inasmuch as the FeO in the surface soil is more than double the amount in the subsoil, it would appear that the reduction effect due to the larger amount of organic matter in the surface soil offsets the oxidizing effect due to aeration.

The presence of aluminum and iron in large amounts favors the formation of insoluble phosphates of these elements. This may explain why phosphorus is the limiting factor in these soils.

Manganese remains about constant in the surface ,subsurface and subsoil.

Titanium oxide ranges from 0.4 per cent to 1.5 per cent in the chief soil types in this country; it is, therefore, relatively low in this soil.

It would appear that the sulfur present is sufficient for present needs; but much of it is combined in such a state, probably in organic combinations, that is not readily soluble in acid.

There is no evidence of the presence of sulfides such as pyrite or marcasite.

It is remarkable that lead and zinc, found in a number of localities in both Siluro-Cambrian and later limestone formations, were not present even in traces in this soil.

Analysis of more or less disintegrated dolomite by Knight (46) showed that 10 per cent of the $CaCO_3$ had been removed, and that the $MgCO_3$ had remained relatively stable. If the Ca and Mg in this soil had been derived entirely from dolomite we would have a ratio of $CaO/MgO = 30.4/21.7$. It is obvious from the results of the chemical analysis that a large portion of the calcium must be derived from amphibole, and that a considerable portion of the magnesium is derived from chlorite.

The amounts of chlorine and sulfuric acid are low. If, as experimental evidence seems to indicate, these radicals are necessary for the synthetic processes in plants or as carriers of the alkalies from the soil to the plant, fertilization with chlorides and sulfates may have considerable effect in this experiment.

This soil is naturally rich in potash, and inasmuch as the orthoclase fragments were found by the micro-petrographical determination to be highly kaolinized and broken up, this may explain the reason why potash dressings alone cause little or no crop increase on this soil type.

THE MINERALS OF THIS SOIL

Chamberlain and Salisbury (9) and Delage and Lagatu (22, 23) were the first to show that nearly all the common rock-forming minerals are to be found in any ordinary soil, formed in the process of weathering and presenting clear unaltered faces. They recognized, also, the result of metamorphic changes, especially in the feldspars, where alteration products could be recognized in the mineral fragments.

The chemical analysis indicates the total amount of elements present in a soil; the mineralogical analysis, however, helps to show in what way these elements are combined.

Considerable work has been done on the solubility of minerals such as the feldspars, micas, tourmaline, chlorite, orthoclase, muscovite, dolomite, limestone, rutile, quartzite, etc., all of which are measurably soluble in water. For details the reader is referred to the work of W. B. and R. E. Rogers (65), Skey (66), Beyer (4), Daubree (21), Clarke (10, 11, p. 532), Cushman (16, p. 6–7) and Funk (33). Cushman's work is of interest as showing that fresh particles of siliceous rock form, upon contact with and decomposition by water, a colloid or gelatinous substance that forms a film on contact with their surfaces which possesses great power of adsorption.

THE AVAILABILITY OF SOME OF THE MINERALS PRESENT IN THIS SOIL

The experimental results on the availability of the various minerals in this soil are not clearly defined.

Cushman (17) called attention to a series of experiments with tobacco seedlings which showed that finely ground orthoclase was nearly as efficient a source of potash plant food as the more soluble potash salts. He emphasizes the necessity for improvements in methods and machines for grinding rock. Prianishnikov (59), Fraps (29, p. 5–16) and Bieler-Chatelan (5) conclude from their experiments that plants assimilate very little potash from orthoclase and microcline even when finely powdered. Fraps showed, however, that they removed from 7 to 13 per cent of the potash from the finely ground micas, biotite and muscovite and Prianishnikov's experiments indicated an assimilation by plants of about 17 per cent of the potash from this mineral. Breazale and Briggs (7) showed that the availability to plants of potash in soils derived from orthoclase-bearing rocks is not increased by addition of lime or gypsum.

The author has been unable to find any experimental work on the availability of the other minerals present in this soil.

THE MINERALOGICAL EXAMINATION

The micro-petrographical examinations were made by Professor Arthur P. Honess of the Department of Geology of the School of Mines of the Pennsylvania State College. Examinations were made on separates made by mechanical analysis by the writer. Professor Honess states that owing to the lack of necessary equipment the examination has not been as complete as could be wished.

Surface Soil

In the surface soil the sand fractions were sieved and examined separately.

Very fine sand (0.1 mm.–0.05 mm.). Mixed feldspars about 50 per cent. Quartz about 35 per cent. The microcline occurs in blue-gray cleavage fragments usually fresh and unaltered, although fragments appear at times slightly kaolinized. The remaining 15 per cent represents iron oxides (some specimens of which are magnetic), rutile, chlorite, dolomite and tourmaline, with limonite predominant.

Fine sand (0.25 mm.–0.1 mm.). Quartz is present in larger amounts than feldspar. Many fragments of quartz from this sample are highly colored by iron oxide and frequently enclose particles whith are brown to black in color and sometimes strongly magnetic. This is probably magnetite or ilmenite. The accessory species are the same as in the very fine sand.

Medium sand (0.5 mm.–0.25 mm.). This does not materially differ from the fine sand, except that quartz and dolomite are somewhat more abundant. There is not the variety of accessory species noted in the finer grades.

Coarse sand (1 mm.–0.5 mm.). This fraction is composed of about 85 per cent quartz. It occurs as rounded or subangular grains; but are occasionally almost spherical. Iron oxide is present either as independent grains or as patches adhering to quartz. A search was made for accessories by crushing part of the sample and mounting in canada balsam; no new species, however, were observed. The total absence of feldspars is noteworthy.

Subsoil

Sand. The sand fractions of the subsoil were not sieved into the various grades as in the case of the surface soil, but were examined as a whole. About 40 per cent consisted of quartz and 45 per cent of feldspar. Occasionally fragments of dolomite were found; limonite grains are common and also iron stained quartz. Some of the microcline fragments are well preserved, others are slightly colored by iron and show some slight evidence of alteration. As accessory components: Tourmaline, mica and rutile were observed.

Surface, subsurface and subsoil

Silts. The silts were difficult to determine due to their finely divided state. Some attempt has been made to determine the minerals present; complete analysis has not been possible because of the lack of necessary apparatus. A preponderance of feldspar (microcline) was noted with a little quartz and a variety of accessories among which tourmaline, chlorite, amphibole, and sericite were unmistakably present.

The silts from the surface, subsurface and subsoil do not materially differ from one another, all being rich in feldspar and showing many accessory species. It is of interest to note that the feldspar is essentially microcline. The silts are thus characterized by the absence of altered minerals.

Remarks on the mineralogical examination

From the examination of the surface sand fractions, it is seen that the finer the fractions the richer it becomes in feldspar and in the variety of accessory species, which gradually decrease in amounts as the fractions become coarser, because owing to perfect cleavage they are more easily broken into fragments and are consequently less resistant to mechanical forces than quartz. The major portion of the soil is composed of these two minerals.

No apatite was found in the fraction examined. Obviously, therefore, the phosphoric acid in this soil must occur accidentally in other minerals, or has escaped detection as apatite in the clay fractions.

Although zirconium was found by chemical analysis, zircon was not found in the mineralogical examination. However, Professor Honess believes that zircon exists in this soil, but that its identity is masked through admixture with other species. Moreover, it may occur in the amphiboles and pyroxenes which are the same chemically, but differ in their optical properties. They have the general formula $RSiO_3$ in which R may be calcium, manganese, magnesium, zinc, sodium, lithium, aluminum, zirconium or titanium.

Rutile occurred usually in small needles or twinned forms and in some cases enclosed in quartz.

Tourmaline was found as an accessory in the silts as well as the sands, but in very small quantities. Chemical examination, however, shows that a trace of boron is present.

There was considerable limonite in the surface fractions. Professor Honess estimates that about two-thirds of the accessory species in the very fine sand fraction of the surface soil is limonite, which according to Clarke (11, p. 532) may contain all sorts of admixtures of other substances such as calcium, manganese, potassium, aluminum, sodium, phosphorus.

The mica is present as sericite which is a secondary muscovite.

It is interesting to note that orthoclase, if present as such, must be in very small amounts and highly kaolinized. No fresh unaltered samples were to be seen in any of the fractions. The potash feldspars yield usually potassium carbonate on decomposition. On this matter, Professor Honess has written that, "It has been suggested by some that orthoclase and microcline, being identical in chemical composition and so closely related in physical and optical character, are one and the same species, the microcline character of orthoclase being the result of sub-microscopic twinning. This, according to Iddings, produces a mono-symetric arrangement of the molecules and orthoclase is then characterized by polysymetry, and consequently not different in crystallization from microcline. But an investigation of these species as found in soils more certainly points to a different conclusion. They are both abundant in the soils of this region, or, more correctly perhaps, originated here, and, although the microcline has survived erosion and decomposition through the ages, it is practically left untouched as an erosion fragment, although occasionally fragments appear slightly kaolinized. The orthoclase fragments have long since been destroyed, and its presence now is only indicated by occasional kaolinized skeetal cleavage fragments or its alternative equivalent."

The feldspars, according to Professor Honess, are mixed feldspars of acid character, or albite variety, and may contain sodium, calcium or potash.

Rutile, zircon, tourmaline apparently have little or no plant food value.

OCCURRENCE OF TITANIUM, MANGANESE, AND THE RARER ELEMENTS IN PLANTS

The rarer elements are more widely distributed in plants than is commonly supposed which shows that soil conditions are favorable for their accumulation. Inasmuch, moreover, as these elements are present as relatively insoluble compounds, the question arises as to why and how such relatively large amounts become absorbed and assimilated by plants and as to how these elements affect plants.

Titanium. According to Czapek (18) Aderholdt found titanium in plants as early as 1852, Wait (74) found it in oak, apple and pear woods, apples, cow peas and cotton seed meal; and Geilman (35) in a number of plants including apples in amount equal to 0.0004 per cent of the air-dried material. Geilman found that it accumulated by preference in the assimilating organs, which appears to confirm Mosea's work (56) that titanic acid is of great physiological importance to plants because it acts as an oxidizing catalyst.

Barium. According to Watts (76, v. 1, p. 500), Scheele was the first to mention the occurrence of barium in 1788 in beech trees. It was found by Hornberger (43) also in beech trees. In 1875 Dworzak (26) found 0.089 per cent and 0.26 per cent of barium oxide in the stalk and ear of wheat respectively, that had grown in the Nile Valley. Marsh, Alsberg and Black (51) give the results of a number of determinations of barium in alfalfa, barley, millet and beets collected from various parts of Colorado, Wyoming and Arizona. McHargue (52) discussing the occurrence of barium in the tobacco plant and the sycamore tree, concludes that it is present in combination with organic acids and that since it is contained in the live cells of the higher plants it may function in metabolism. Artis and Maxwell (1) report amounts of barium in the leaves of tobacco, dogwood, cottonwood, cherry leaf, black locust, mulberry, elm, maple, plum, walnut, pear, linden, and box-elder varying from 0.005 per cent BaO in the cottonwood to 1.07 per cent in the mulberry. Headden (39, p. 10–17) found it in alfalfa, corn leaves, pea vines and tobacco grown in Colorada in amounts varying from 0.02 to 0.2 per cent BaO. The writer has found 0.22 per cent BaO in alfalfa grown on the Pennsylvania State College Farm.

Caesium. Von Lippman (73) found caesium in the ash of sugar beets.

Rubidium. Pfeiffer (57) reported rubidium in sugar beets. According to Watts (76, v. 5, p. 127), it has also been found in coffee, tea and cocoa.

Vanadium, chromium and molybdenum. Demarcay (24) identified these elements by the spectroscope in the ash of grapevines, fir and poplar. According to Robinson (62, p. 4), von Lippman found vanadium in the ash of grapevines, sugar beets and various trees.

Lithium. Gaunersdorfer (34) and later Tschermak (68) found lithium in a number of plants and Headden (39, p. 10–17) found it in loco weed, alfalfa and tobacco. The writer has found it in alfalfa grown on the Pennsylvania State College Farm.

Strontium. Headden (39, p. 10–17) found notable amounts in loco weeds, corn leaves and alfalfa. The writer has found 0.10 per cent SrO in alfalfa grown on the Pennsylvania State College Farm.

Boron. Jay (45) found small amounts of boric acid in onions.

Zirconium. The author has not been able to find any mention in the literature of the occurrence of zirconium in plants.

Special mention must be made of the work of Robinson (63, p. 9–17), who has carried out extensive investigations, by methods of unquestionable validity, on the relation of some of the rarer elements in soils and plants. Plants grown on soils in which the rarer elements had been found were examined with the following results:

> In apples he found barium, strontium, lithium, rubidium and a trace of titanium; in beets, chromium, vanadium, barium, strontium, lithium, rubidium and caesium; in alfalfa, barium, strontium, lithium and rubidium; in beans, lithium and rubidium; in cabbage and bluegrass, a trace of chromium; in corn, timothy and wheat, titanium, rubidium, strontium, lithium and barium; and in the case of timothy, chromium and caesium.
>
> He concludes that lithium is present in traces in all plants examined and that rubidium is present in the majority of cases in amounts ranging from a trace to 0.005 per cent of the dried material, which is larger than the amounts present of the other rare alkalies. Caesium was found in three plants. Chromium was found in amounts ranging from a trace to 0.004 per cent of the material. Vanadium was found in traces in six plants. Barium was found in all cases and strontium in all samples except bean seed. Molybdenum was not found in any samples.

It is of interest to note in connection with Robinson's work that where the plant contains a comparatively large amount of the rare alkalies there is also an abnormal amount of manganese present.

THE EFFECT OF THE RARER ELEMENTS ON PLANTS

Very little experimental work has been carried out on the physiological action of the rarer elements on plant tissues.

That lithium has a toxic action is shown by the work of Gaunersdorfer (34), Voelcker (71, 72), Frerking (31) and Ravenna and Zamorani (60). Voelcker found that the chloride and iodide applied at the rate of 2 cwt. per acre tends to check root growth in the case of barley, wheat and corn; in some cases actually destroying a proportion of the plants grown; but they found that the application of the oxide at the same rate had a beneficial effect on wheat and barley. Frerking found the poisonous influence of lithium more pronounced than of magnesium in organisms requiring calcium, but not to calcium-free algae and fungi. Very little work has been done on the effect of caesium; but the work of Lucanus (50) and Voelker showed that its salts are less harmful than the salts of lithium.

McHargue (53) states that, although it has been known for more than a century that plants are able to extract appreciable amounts of relatively insoluble compounds of barium contained in soils, little has been done to determine whether or not the compounds of this element have any specific function in vegetable economy. He showed that barium compounds in the absence of calcium carbonate are poisonous to plants; but barium carbonate in the presence of calcium carbonate apparently exerts a stimulating influence.

Haselhoff (38) found that strontium had no injurious action, is absorbed by plants, and the results indicate that it replaces calcium when there is an insufficient supply of that element. McHargue's (53) results, however, show that $SrCO_3$ cannot be substituted for $CaCO_3$. In Loew's (49) experiments on algae, strontium salts injuriously affected the chlorophyl bodies and caused loss of starch-making power and finally death. The physiological effect of rubidium chloride has also been investigated by Loew (48), who in pot culture experiments with Chinese barley, cabbage and spinach found that there was a stimulating action in amounts not exceeding 10 mgm. per 50 kgm. of soil; but when applied at the rate of 50 mgm. per 50 kgm. of soil the stimulating effect was not so great except in the case of cabbage as when small quantities were used.

Hebert (40) tested the effect of the sulfates of chromium, aluminum, magnesium, zirconium, thorium, cerium, lanthanum, etc., on germinated seeds of peas, wheat, rape and the yeasts. The toxicity of the different compounds varied slightly on the different groups, but in general occurred in the following diminishing order: Zirconium, thorium, aluminum, chromium, cerium and magnesium. With regard to vanadium, Ramirez (cited by Robinson, 63, p. 9–17) found that it is absorbed and stored by some plants and causes anomalies in their growth. A review of the literature of the presence and effect of boron in plants is given by Cook (12) and Cook and Wilson (13, 14). It would appear that numerous factors influence the absorption, distribution and action of boron on plants. There is evidence to show that below a certain dilution boron tends to produce stronger roots and shoots. This stimulating action appears to be due to the increase of nitrates. Large amounts are known to be toxic to all plants with the exception of certain fungi.

A fairly complete review of the effect of manganese is given by Dietrick (25).

Summarily it would appear that each ash constituent must be considered as exerting some slight influence on the plant. Though plants may develop normally in a nutrients olution without a given element, it does not necessarily follow that this element, if present, might not exert some influence, for the absence or presence of any particular element might be the deciding factor in determining whether or not the plant reaches full development.

Elements like titanium, manganese, barium, boron, strontium, rubidium, etc. may act as catalyzers.

SUMMARY

This introductory paper gives an account of the preliminary work carried out on the soil used in an intensive apple fertilizer experiment conducted in steel rims as an Adams Fund Project. At the present time it is desired to draw attention to the following main facts.

1. The rarer elements vanadium, chromium, molybdenum, zirconium, rubidium, caesium and lithium have been isolated and identified by qualitative and quantitative methods of indisputable validity from a residual soil remote from metamorphic rocks.

2. Positive proof is presented of the presence of barium and strontium, which is the first announcement made of the occurrence of these elements in this phase of the Hagerstown series of soils. The results for BaO are believed to be accurate. However, as will be seen, the results for SrO are approximate. for complete separation from calcium was not obtained.

3. The calcium and magnesium are derived not only from dolomite, but a considerable portion of the quantity of these elements present are contributed by the silicates amphibole and chlorite respectively. Though this soil is formed from limestone rocks it contains no $CaCO_3$. Moreover, magnesium is present in larger amounts than calcium.

4. The chief source of potassium in this soil are microcline and the mixed feldspars of acid variety, on the availability of which contradictory evidence has been obtained by different experimenters. Mica is present only as an accessory. The orthoclase has practically all been kaolinized.

5. The existence of iron in the ferrous condition, notwithstanding difficulties in determining the absolute amounts, has been definitely established.

6. The greater portion of the sulfur exists either in the free state or, as seems most probable, in organic combination.

7. All three soil layers contain only a trace of chlorine (chlorides) which is most certainly remarkable for it is almost invariably present in soils to the extent of about .01 per cent.

8. Accurate methods for the determination of boron and fluorine in soils need to be developed.

9. A review of the literature of the occurrence of the rarer elements in plants and of the experimental work on the effect of some of them is given. The question is raised—how do such relatively large amounts of the rarer elements become absorbed and assimilated by plants, and what function, if any, do they possess?

As this experiment develops it is planned to investigate the absorption not only of the main plant food elements, but also of some of these rarer elements and the author hopes that the presentation of the facts in this paper will stimulate work along similar lines by others. It is fully realized that a new set of variables is presented in our already complex soil problems; but it is only by a correlation of all variables that an advance will be made in our knowledge of these formidable biological problems.

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THE EFFECT OF THE SPRAY PROCESS OF DRYING ON THE VITAMIN C CONTENT OF MILK

A PRELIMINARY REPORT *

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On account of the conflicting statements in the literature regarding the vitamin C content of dried milk products, and especially in view of definite statements regarding the effect of the spray process on vitamin C, a special investigation of this problem has been conducted.

This investigation was started in February, 1922. The method consisted in feeding young guinea-pigs a standard basal diet free from vitamin C. To this was added raw liquid milk or spray process powdered milk in varying amounts, these milk products being the only source of vitamin C in the diet.

The milk, both liquid and powdered, was shipped by express daily from the Merrell-Soule Powdered Milk Factory at Perry, N. Y. The milk, both raw and powdered, which was used in this experiment was taken from the mixed milk of 175 dairies representing 1,276 cows. The raw milk for feeding was drawn each day from a large vat. In order to prevent souring of the raw milk, it was cooled and shipped in sterilized vacuum bottles. The milk in the vat was pasteurized under conditions known to be favorable to the preservation of vitamin C and then reduced to powder. The powdered milk for feeding was drawn each day from this powder so that the raw milk and powdered milk came from the same lot of fresh milk. Unless this precaution is taken, a fair comparison between raw and powdered milk cannot be made, as the vitamin C content of different lots of fresh liquid milk has been found to vary considerably. The experiments on winter milk were kept separate from those on summer milk on account of a similar variation.

This difference between summer and winter milk was confirmed by the present experiment. The fact indicates that no reliable conclusions regarding the effect of drying processes on the vitamin C content of milk can be drawn unless comparison is made between the dried milk and fresh raw milk taken from the *same* lot. Failure to take this precaution may probably account for some of the contradictory reports previously published.

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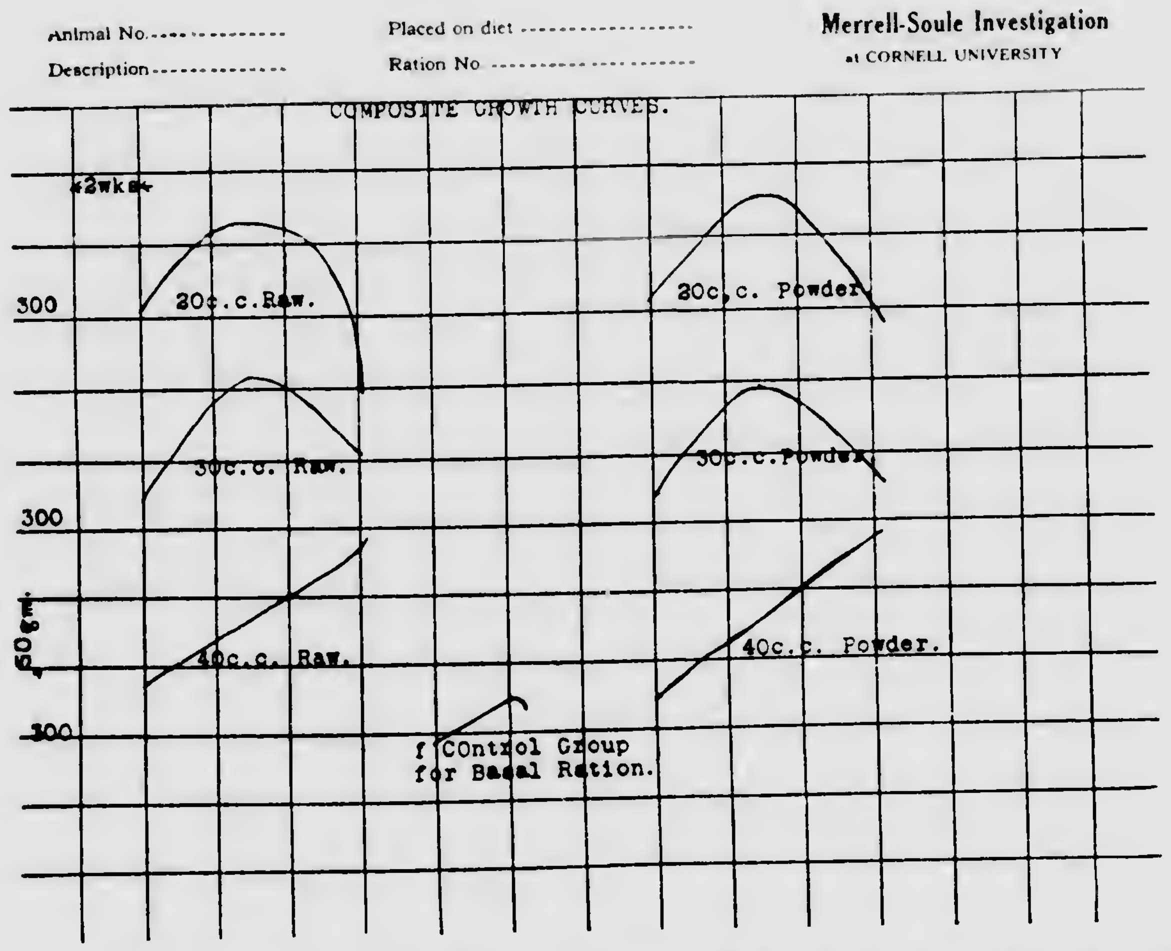
* This investigation was carried on at the New York State College of Agriculture at Cornell University by George W. Cavanaugh, Professor of Agricultural Chemistry of Cornell University, and James S. Hall, under the direction and advice of Prof. R. Adams Dutcher, Head of the Department of Chemical Agriculture of the Pennsylvania State College.

PLAN OF EXPERIMENT

The guinea-pigs were arranged in groups according to the amount of milk fed, with eight or nine animals in each group. Each animal received, daily, the basal ration plus the milk to be tested, some groups receiving raw milk, others equivalent amounts of the powdered milk. The powdered milk was redissolved in water before being measured out for feeding, thus restoring it to the same water content as that of the raw milk.

Careful records were kept for each animal showing daily basal ration intake, daily milk intake and body weight; also, date of development of scurvy, date of recovery from scurvy or date of death, when these events occurred. In each case of death, a necropsy was performed. This part of the experiment, in which 350 animals were used, extended over a period of seven months.



Curves showing average growth of guinea-pigs in the six experimental groups and in control group.

A careful study of all the groups, taking into account length of life, rapidity of growth, time of onset of scurvy and other factors indicated that milk, powdered by this spray drying process, was not inferior in antiscorbic properties to the original raw milk. In other words, the result did not show any evidence of the destruction of vitamin C in milk powder prepared by this process.

The results of this part of the work were complicated by an epidemic of influenza in the colony, so that many animals had to be eliminated. There was also a lack of uniformity in the behavior of the animals within each group, owing apparently to the poor physical condition of many of them when received. These animals were obtained from three or four different breeders, which may in part account for the lack of uniform condition.

The experiment was, therefore, repeated. Special precautions were taken to obviate the foregoing difficulties. These may be of interest because a marked improvement in the uniformity of the results within each group and a consequent decrease in the experimental error were obtained.

All the guinea-pigs for the repetition of the investigation were obtained from the same source. Young animals were chosen from mothers that had been kept under identical conditions. These young animals had received the same food since birth. Healthy guinea-pigs weighing from 210 to 280 gm. were selected. Each one was placed at the caviary in an individual cage with some green food. They were then transported by hand to Cornell University, reaching their destination within twelve hours.

There these guinea-pigs were kept under observation for three weeks, and those that failed to grow normally were discarded. During this preliminary feeding period they received the following diet: a basal ration consisting of 60 per cent. of ground oats; 15 per cent. of wheat bran; 15 per cent. of ground autoclaved alfalfa; 5 per cent. of casein; 1 per cent. of calcium carbonate; 1 per cent. of sodium chlorid, and 3 per cent. of butter fat. In addition to this basal ration, whole milk and green grass were fed ad libitum.

At the end of the three weeks' observation period, the animals that showed normal growth curves were divided into six groups. Each group consisted of ten animals. The animals in each group received the basal ration ad libitum. In addition, they were given different amounts of raw whole milk and reconstituted Klim brand Merrell-Soule powdered whole milk according to this plan: Group 1, 20 c.c. of raw whole milk; Group 2, 20 c.c. of reconstituted milk powder; Group 3, 30 c.c. of raw whole milk; Group 4, 30 c.c. of reconstituted milk powder; Group 5, 40 c.c. of raw whole milk, and Group 6, 40 c.c. of reconstituted milk powder.

A control group of five animals received the basal ration with water ad libitum. Previous studies on the antiscorbutic properties of milk have shown that 30 c.c. is about the minimum amount that will protect guinea-pigs from scurvy. For this reason, levels of 20, 30 and 40 c.c. of milk were used in this experiment.

RESULTS

December 26, the sixty-first day of the experiment, the results showed that the average number of days for the onset of scurvy in groups fed raw and powdered milk are closely comparable.

The data obtained are given in the accompanying table.

Only five guinea-pigs of Group 5 developed scurvy, four of which have recovered. Only three guinea-pigs in Group 6 developed scurvy, all of which have recovered.

For this reason, no average time of onset of scurvy is given for these groups.

It was noted that the basal ration was apparently adequate, as the animals gained weight even while developing scurvy. This seems to eliminate complications only too common in this type of experiment in which incomplete diets are used for basal rations.

The present part of this experiment is now in its sixty-first day. It will be continued for a total period of at least ninety days. A preliminary report is made at this time because the results seem to be concordant and to confirm the earlier and more extended part of the work.

A study of the groups at this time shows that the control group developed scurvy more quickly than the groups fed raw or powdered milk. Each group fed powdered milk did at least as well as the group receiving an equivalent amount of raw milk. So far as we can judge from data obtained from these experiments with guinea-pigs, there appears to be no destruction of the antiscorbutic properties of milk when it is thus powdered. This confirms the conclusions drawn from our first experiment; i. e., that milk powdered by this form of spray process possesses as great antiscorbutic properties as the raw milk from which it was made.

Results in Groups Fed Raw and Powdered Milk

	Average Weight at Beginning, Gm.	Average Maximum Weight, Gm.	Average Gain, Gm.	Average Time for Onset of Scurvy, Days
Control group	296	319	23	14.8
Group 1—20 c.c. raw milk	308.9	362.2	53.3	18.4
Group 2—20 c.c. powdered milk	310.7	376.1	65.4	20.3
Group 3—30 c.c. raw milk	319.6	402.6	83.0	21.1
Group 4—30 c.c. powdered milk	317.1	395.2	78.1	22.8
Group 5—40 c.c. raw milk	338.7	434.0	95.6	
Group 6—40 c.c. powdered milk	326.1	444.0	118.0	

CONCLUSIONS

The results of these experiments to date indicate quite clearly that the spray process of drying milk preserves in its original strength the vitamin C; i. e., the antiscorbutic properties of milk.

The experiment is being continued to determine the effect of aging on the vitamin C content of powdered milk. So far, results indicate that spray process powdered milk probably retains its antiscorbutic properties for many months. Should this point be confirmed by the final results, its importance will be obvious. Summer milk, notably rich in vitamin C, could be powdered, and this powder could be used for infant and child feeding in the winter months, at which time raw milk is notably deficient in this vitamin.

FACTORS INFLUENCING THE VITAMIN CONTENT OF COW'S MILK.

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Lunin (1), in 1881, made what was probably the first suggestion that milk contains substances other than proteins, fats, carbohydrates, salts and water which are indispensable for normal nutrition. This statement was based upon the fact that he was not able to rear experimental animals successfully on a ration containing apparently adequate amounts of proteins, fats, carbohydrates, and salts. Small additions of milk, however, caused these animals to develop in a normal manner· Little attention was paid to Lunin's suggestions, however, until 1906, when Hopkins (2) reported "that no animal can live upon a mixture ef pure protein, fat and carbohydrate and even when the necessary inorganic material is carefully supplied, the animal still can not flourish. The animal body is adjusted to live either upon plant tissues or other animals, and these contain countless substances other than proteins, carbohydrates, and fats." In 1909, Stepp (3) reported that bread and milk was a satisfactory diet for rats. When, however, he extracted the mixture with alcohol and ether, the extracted residue would not longer support growth. Addition of the ether-alcohol extract stimulated growth when animals had failed to grow on the extracted residue. This work added further evidence to support the idea that milk contains unknown growth-promoting factors. Later, Hopkins (4) published data to show that 2 cubic centimeters of milk were sufficient to supply enough of the unknown growth-promoting materials for growth.

Since that time experimental data have accumulated until practically all nutrition authorities are agreed that an important part of the nutritive value of milk lies in the amount of the vitamins A, B, and C present at the time that the milk is ingested. It is now becoming quite evident that a fourth factor, the antirachitic vitamin, must be considered in all vitamin studies. Any information, therefore, that we can obtain concerning the factors which influence the vitamin content of milk will be of considerable value. The factors considered in this paper are (*a*) diet, (*b*) heat treatment, and (*c*) oxidation.

MILK VARIES IN VITAMIN CONTENT.

In 1920, Osborne and Mendel (5) published data to show that 2 cubic centimeters of fresh milk would not suffice for growth when fed as the only source of vitamin B, and, what was more interesting, rats did not grow normally until the amount of milk was increased to 16 cubic centimeters. Kennedy and Dutcher (6) made similar observations, with the exception that 10 cubic centimeters of milk carried sufficient vitamin B for growth. These and similar observations with reference to vitamins A and C, naturally raised the question in the minds of many investigators as to the factors causing the variability in the vitimin content of milk. The variations were particularly striking with regard to the antiscorbutic properties of milk. Chick, Hume, and Skelton (7), for example, reported that 85 cubic centimeters (daily) of milk were necessary for the protection of guinea pigs from scurvy, while other investigators reported prevention of scurvy in guinea pigs with amounts of milk varying from 50 to 130 cubic centimeters.

THE INFLUENCE OF DIET.

A number of investigators noted the variability in vitamin potency of cow's milk obtained from various sources and, although experimental proof was lacking, it seemed probable that the vitamin content of the diet might be an influencing factor. Funk (8) suggested this possibility as early as 1914. Previous to Funk's observation, Andrew (9) had reported that babies, nursed by mothers afflicted with beri-beri, also became afflicted with the disease. McCollum and his coworkers (10) arrived at similar conclusions while conducting feeding experiments with rats. As we (11) have pointed out in a previous paper, the evidence obtained by McCollum, with rats, was not conclusive due to the fact that he was unable to say with certainty that the nursing rats did not grow because of the vitamin deficiency of the mother's milk. He had no way of measuring the milk flow of the mother rats, and it was possible that the young were starved due to decreased milk flow.

In August, 1919, Dutcher, Pierson, and Biester (12) announced that they had noted a seasonal variation in the antiscorbutic potency of milk. A similar observation was made simultaneously by Barnes and Hume (13). In 1920, Hart, Steenbock, and Ellis (14) described experiments in which milk from the university herd was compared with milk obtained from cows which had been fed for a number of years on air-dried roughages and grains. These writers found that the milk from the latter herd was much inferior in antiscorbutic potency to the milk obtained from the university herd. During the same year Dutcher, Eckles, and coworkers (11) described experiments which differed from the Wisconsin work in that the experimental milk was obtained from the same cows, thereby eliminating any possibility of introducing variations due to differences in individuals.

In the last-mentioned study two cows were fed a vitamin-poor ration from January 17, 1920, until June 1, 1920. On June 1 the cows were given access to fresh pasture grass and continued on the experimental ration until October 1, 1920. During the vitamin-poor and vitamin-rich periods, groups of guinea pigs were placed on a basal ration of oats and varying quantities of the experimental milk. After feeding a relatively large number of guinea pigs for the eight months' feeding period it was concluded that 20 cubic centimeters of the "summer milk" was superior to 60 cubic centimeters of the "winter milk." It was found that there was a tendency for the milk to become poor slowly when the diet of the cows was deficient in vitamins, while the nutritive value of the milk became relatively high almost immediately after the cows were given access to green grass. In other words, it would seem that Mother Nature uses this method to protect the young by allowing the nursing mother to dole out her vitamins sparingly from her body tissues in order to keep the milk adequate. On the other hand, the milk becomes vitamin-rich rapidly after a change to a vitamin-rich diet. Simultaneously with the last-mentioned work, Hess, Unger, and Supplee (15) reported similar observations regarding milk powders made from milk obtained from cows fed in a similar manner. The milk powder, obtained when the cows were fed a vitamin-rich ration, was superior in antiscorbutic properties to milk obtained from the same cows during a vitamin-poor feeding period.

Previous to the publication of the last-mentioned articles, Osborne and Mendel (16) had announced that they were unable to find that summer milk was superior to winter milk as far as vitamin B was concerned. This can be explained on the basis that it is almost impossible, under ordinary conditions, to feed dairy cows a ration that is not fairly rich in this vitamin. It is reasonable to expect that milk could be produced which is practically devoid of vitamin B, provided a sufficiently poor ration be employed. Hughes, Fitch, and Cave (17) reported in 1921 that they were unable to demonstrate that the antiscorbutic potency of milk could be affected by diet although vitamins A and B were present in greater quantity when the cows were fed a vitamin-rich ration.

In 1922, Kennedy and Dutcher (18) announced the results of their work on the influence of diet upon the quantity of vitamins A and B in milk. It was concluded that the amount of vitamins A and B in milk are directly dependent upon the vitamin content of the dairy ration and that stall-fed cows will produce a vitamin-rich milk if fed the proper combination of grains, silage, and legume hay.

It is reasonable to believe that the principles established in the researches just reviewed will apply to the feeding of all lactating animals. Medical men have known for a great many years that, other things being equal, infants thrive best when the diet of the mother is adequate and varied.

THE EFFECT OF HEAT TREATMENT.

It is impossible in a paper of this type to include all of the work that has been done in this field. The speaker will be satisfied if he is able to outline the general trend of events and indicate some of the more important tentative conclusions that might be drawn.

As early as 1894 Barlow (19) was convinced that "cooked" milk was inferior to raw milk in antiscorbutic potency. Without attempting to review the literature relating to the clinical observations made by physicians and nutrition workers, it will suffice to say that many practitioners contended that Pasteurized and "cooked" milk should be avoided, due to the fact that scurvy invariably developed. Other authorities, among whom the name of Lane-Claypon (20) is prominent, contended that the nutritive value of milk is not injured by heating.

Since the advent of the vitamin hypothesis considerable work has been done with the view of determining the stability of the various vitamins to heat. Hess and Fish (21) stated in 1914 that heat slowly destroyed the antiscorbutic properties of milk, but that an intense heat for a short period seemed to be less destructive than a lower temperature for a longer time. Barnes and Hume (22) reported that about half of the antiscorbutic potency was lost when milk was heated rapidly to the boiling point over a gas burner. Hart, Steenbock, and Smith (23) have shown that the antiscorbutic vitamin is destroyed when milk is heated at 120° C. for 10 minutes.

Miss Edla Anderson, working in the speaker's laboratory, has shown that Pasteurization of milk in closed bottles at 145° F. for 30 minutes does not destroy appreciable amounts of vitamin C. When this Pasteurized milk was fed to guinea pigs at 30 cubic centimeter levels as the source of vitamin C, there was little if any difference between the animals and those fed an equal quantity of raw milk. This agrees with the observations of French pediatritians (24), who report the feeding of thousands of babies on milk, Pasteurized in bottles, without a single case of scurvy. It has been our experience that, as a rule, 30 cubic centimeters of milk will protect 250-gram guinea pigs for periods of 90 to 120 days, providing the milk is reasonably rich in vitamin C. This being the case, we have adopted the practice, in our experimental work, of using 20, 30, and 40 cubic centimeters of milk in our scurvy studies; 20 cubic centimeters will not protect for long periods, while 40 cubic centimeters is ample if the milk is rich in vitamin C. It has been our experience that 30 cubic centimeters of milk represents the "threshold" quantity of vitamin C for scurvy studies. For that reason we have felt that any study involving the effect of heat or oxidation should include groups of animals receiving about that quantity of milk in addition to the basal ration. Any slight destruction will make itself evident when milk is fed at this level. We have also found it to be absolutely necessary that control animals be fed at the same time on equal quantities of untreated raw milk.

While the antiscorbutic vitamin is probably the most susceptible of all of the known vitamins to heat, nevertheless it would appear that the ordinary methods of vat Pasteurization as practiced in this country are less destructive than has been thought. We have fed guinea pigs Pasteurized milk that was made by the holding process (145° F. for 30 minutes) that compared quite favorably with the same milk before treatment. It is true that heat has a destructive action on vitamin C, but the advantages of Pasteurization more than counterbalance the disadvantages due to vitamin destruction. It should be remembered that milk is not considered a vitamin-C-rich food, and for that reason it is the practice of progressive physicians to prescribe orange juice or tomato juice for bottle-fed infants. In this connection it should be emphasized, also, that milk is diluted in preparing the modified milk used in infant feeding. This often lowers the vitamin potency to the danger point.

While we are discussing the vitamin content of milk from the standpoint of infant feeding, it should be pointed out that the recent work of McCollum and coworkers at Johns Hopkins University has shown quite conclusively that cod-liver oil contains two vitamins, vitamin A and an antirachitic factor. It has been known for some time that rickets is most prevalent in bottle-fed infants indicating that milk is not particularly rich in the antirachiti substance. For that reason, prominent pediatritians (25) are recommending the feeding of cod-liver oil as well as orange juice to bottle-fed normal healthy infants.

When we consider the question of the destruction of vitamin A in which milk is relatively rich, we find practically no data concerning the destruction of this vitamin in the heating of milk. Osborne and Mendel (26) passed live steam through butterfat for two and one-half hours. Rats grew well on a ration containing 18 per cent of this heated butterfat. These authors concluded, therefore, that vitamin A was stable to heat at the temperature of steam.

Later, Steenbock, Boutwell, and Kent (27) showed that butter kept at 100° C. and stirred by a stream of air for 12 hours would not produce good growth. In 1920, Osborne and Mendel (28) published data confirming their previous work. It soon became evident that oxidation was playing a part in the destruction of vitamins by heat.

OXIDATION.

In 1919, Zilva (29), in working with ultra-violet rays, noted the destruction of vitamin A in butter when the butter was exposed to the rays for several hours. He noted the presence of ozone, and further experimentation (30) brought to light the fact that the destruction of vitamin A was due to oxidation. He also was able to destroy this vitamin in cod-liver oil, although it required more severe treatment due to the high concentration of the vitamin in cod-liver oil. In 1920, Hopkins (31) and Drummond and Coward (32) announced the destruction of vitamin A in butterfat by atmospheric oxidation.

The following year Drummond, Coward, and Watson (33) published the results of their work on milk and butter and showed that colostrum was richer in vitamin A than the later milk. They reported a loss of vitamin A in butter manufacture, due in all probability to the fact that a portion of this vitamin is in the milk serum. They also emphasized the importance of the diet of the cow with regard to the vitamin content of the butter.

While conclusive evidence is lacking, it would appear that there is little to fear in the Pasteurization or boiling of milk, so far as vitamin A is concerned, for the vitamin appears to be stable to heat up to 100° C., and the opportunity for excessive oxidation in ordinary milk treatment is not great.

There is little evidence that ordinary methods of Pasteurization or boiling have a destructive action on vitamin B. In fact it would appear that this vitamin is relatively stable to temperatures up to 100° C. Daniels and Loughlin (34) have shown that the nutritive value of milk is impaired by heating, but that the loss of nutritive value is due to the loss of calcium and phosphorus salts rather than a vitamin destruction. Mr. J. R. Haag, working in my laboratory, has fed rats a diet consisting solely of raw and Pasteurized milk, but has been unable to find that the milks differed in nutritive value.

McCollum oxidized cod-liver oil by bubbling air through the oil at 100° C. and was able to destroy vitamin A, but the antirachitic factor was unimpaired. It is evident, therefore, that the antirachitic vitamin is stable to heat and oxidation. Little is known, however, at the present time, regarding the distribution of this vitamin in other foodstuffs.

When we consider the effect of oxidation on the antiscorbutic vitamin, we find that we are dealing with a less stable substance. Hess (35) showed in 1920 that milk lost some of its antiscorbutic properties when shaken with air. Miss Anderson, working in my laboratory, observed (36) that vitamin C was destroyed when milk was violently agitated in an open dish during Pasteurization, while the same milk Pasteurized in closed bottles compared favorably with the untreated milk. When air was bubbled through the milk during Pasteurization some destruction occurred, but the destruction was small compared to that obtained when oxygen was used instead of air. Oxygen and hydrogen peroxide destroyed this vitamin at room temprature, although the destruction took place more rapidly on heating. When milk was agitated with a stream of carbon dioxide during Pasteurization, there was little if any destruction of vitamin C.

While it is not within the province of this paper to discuss the vitamin content of powdered milks, it is of interest to note that many of the commercial milk powders made by the spray process (37) have been found to be deficient in vitamin C, while powders made by the roller process seem to be somewhat superior (38).

Experiments just recently terminated at Cornell University indicate that the present methods of making sprayed whole-milk powder as now in use at the Perry, N. Y., plant of the Merrell-Soule Co., do not destroy the antiscorbutic potency of the milk. In these experiments raw whole milk and milk powder were shipped daily from the plant to the university. The powdered milk was made to the same concentration as the raw milk by the addition of water, and the milks were compared, using guinea pigs as the experimental animals. The results show no differences between the raw and the powdered milks. This is explained by the fact that the milk is condensed in vacuum, and the condensed milk is Pasteurized without agitation in a closed container in the absence of air. This milk is then sprayed at an enormous pressure into a "hot room," and the atomized particles are dried instantly and removed in a relatively short time. It would appear, therefore, that it is possible to make sprayed milk powder without destroying vitamin C, and that the spraying operation does not of itself destroy this vitamin, provided the process is properly regulated.

As I have pointed out in a previous article (39), "even if it were proven, eventually, that condensed milks and milk powders were devoid of vitamins—which I doubt—there would still be a very important place for these products in the diet when supplemented properly by other foods. It would be just as inadvisable to make sweeping condemnations of these important commodities as to condemn white corn as a food because it appeared to be somewhat inferior to yellow corn. What we should try to do is to get at the facts, prove conclusively that they are facts, and then take steps to improve methods of manufacture and to supplement our deficient foods with those which are known to be rich in vitamins."

While milk is the most valuable single article of food that we have, let us recognize that it is not, as many would try to make us believe, a perfect food. In spite of the fact that I have pointed out certain deficiencies that may occur in milk, let us not forget that we should do all in our power to make the public appreciate that there is no food that can take the place of milk, and that increased consumption of milk, supplemented with other foods, should be encouraged.

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96 **(2328)**

Vitamin studies. X. Feeding technique in vitamin studies.*

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The work described in this paper is presented with the view of re-emphasizing the importance of the use of false screen bottomed cages in vitamin studies when rats are used as the experimental animals. Steenbock, Sell and Nelson[1] have pointed out that rats which do not have access to their own feces require a larger proportion of vitamin B in the basal ration than those rats which ingest variable amounts of excretory material daily. In our studies of vitamin B in the past (using diets deficient in this vitamin), we have often been unable to account for the lack of uniformity in the development of animals from the same litters on identical diets. In fact certain animals grew satisfactorily for many weeks on a vitamin B deficient ration. In watching the animals over relatively long periods it was observed that those animals whose food intakes and growth curves were most satisfactory were in the habit of ingesting relatively large amounts of excretory material; for example we have noted a few animals which would consume as many as 15 to 20 pieces of feces in the space of eight or ten hours.

In order to study the matter more thoroughly 63 rats were placed on experiment in individual cages. All animals received a basal ration consisting of casein 18, salts 3, agar 2, dextrin 77 and 5 drops of cod liver oil daily. Iodine was furnished in the drinking water. A number of the animals were placed in cages without screens while the remainder were distributed in cages containing false bottoms made of galvanized wire screens (3 meshes to the inch). The false bottoms were constructed about 1 1/4 inches above the cage pans in order that the particles of feces would fall through the screens to the pans below where the rats could not reach them. As indicated in the chart, weekly records were taken of the amount of food eaten, but it was impossible to keep an accurate record of the weight of fecal material ingested. In order, however, that we might have a fairly definite idea of the feces intake, certain rats were placed on screens, and feces from definite sources were fed. In some cases the feces were collected from rats which were on diets rich in vitamin B, while in other cases feces were collected from rats on diets containing little or none of this accessory food substance.

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[1] Steenbock, H., Sell, M. T., and Jones, J. H., *J. Biol. Chem.*, 1923, lv, 399.

Some of the animals were placed on screens from the start and later the screens were removed in order to provide ready access to the fecal particles. Other rats were allowed access to feces from the start and later the screens were introduced.

In order to conserve space we have chosen a few of the most striking curves, placing them in a single chart for comparison. Certain curves were chosen, not because they are necessarily representative, but rather to show to what extremes animal experiments may go when seemingly unimportant details in technique are neglected.

The curve given for rat 392 is representative of all animals which were on screens without access to feces from the beginning of the experiment. It is evident that there is little, if any, vitamin B in the basal ration. When vitamin-rich feces from another rat were fed to rat 392 (see arrow) the response in food intake and growth was immediate. Although rats 292, 293, 296 and 299 received the same ration as rat 392, it is interesting to note that they were able to grow at a fairly satisfactory rate for from five to seven months, in spite of the fact that the ration was deficient in vitamin B. When screens were introduced in the cages containing rats 292 and 293, the fall in the food intake and body weight was very marked. On the 230th day vitamin-rich feces, obtained from rats on a vitamin-rich diet, were placed in dishes and offered to rats 292 and 293. The increase in food intake and body weight is ample proof that the feces possessed a stimulatory effect.

The curves of rats 299 and 301 tell the same story up to the point where the screens were introduced. When vitamin-rich feces were fed, the responses were very marked while the reverse was true when vitamin-poor feces were fed (see curve for rats 296, 299 and 301). The parallelism between food intake and increase in body weight is interesting and emphasizes the neces-

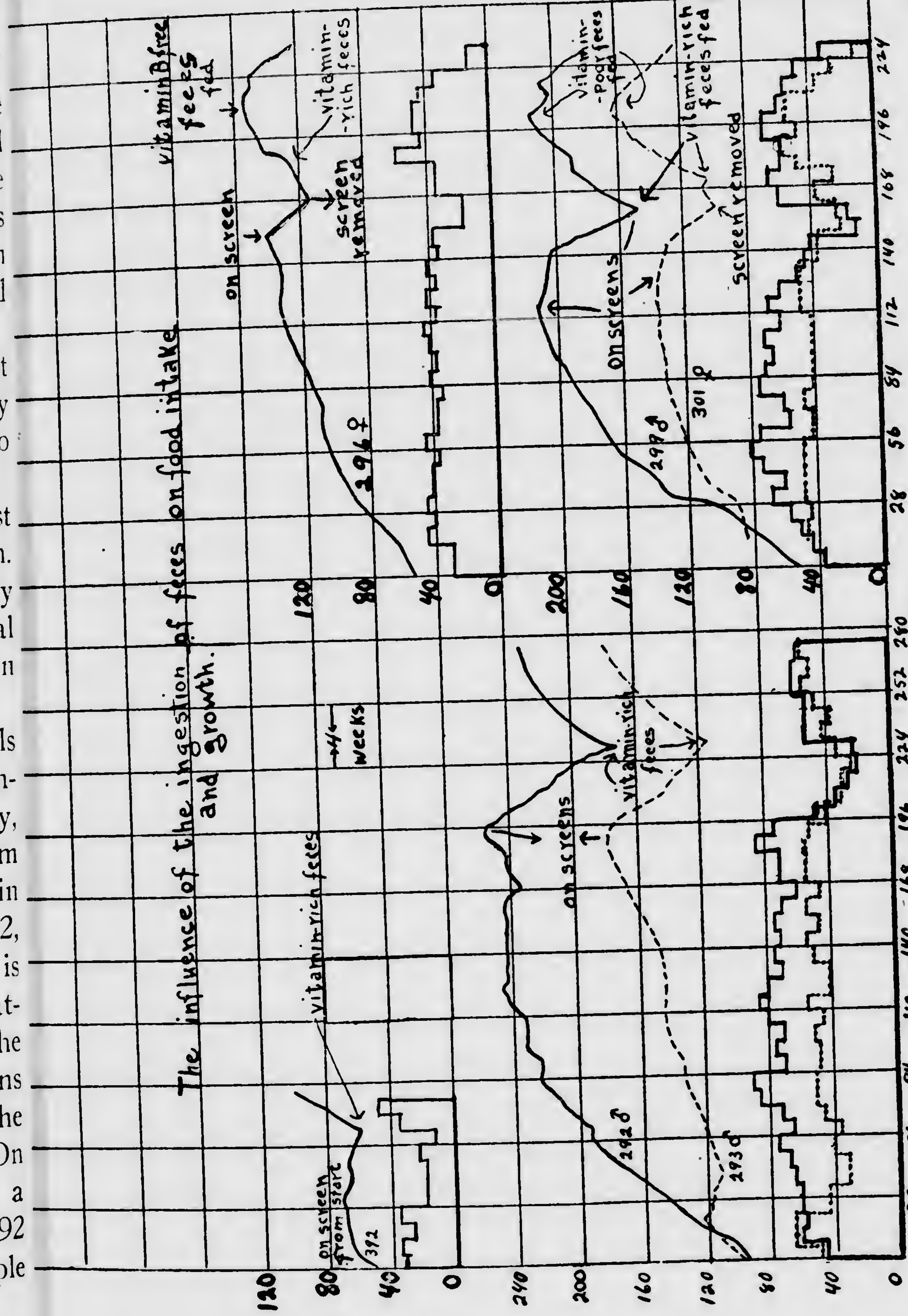

Chart showing the deportment of albino rats on diets deficient in vitamin B, with and without the use of false screen bottoms in the cages. Those rats which consumed large quantities of excreta were able to grow well—due, apparently, to the tissue vitamin stored in the body previous to the feeding trials. It would appear that this food hormone can be used over and over again. When rats are placed on screens appetite and body weight fall almost immediately. Rats cannot store vitamin B for long periods. This is indicated by the fact that the feces of the rats (on screens) have lost this vitamin within two weeks time.

sity of possessing data on food intake of individual animals. This, in our opinion, has considerable advantage over the "group feeding" method inasmuch as it often aids in the interpretation of data. For example we have found it advisable to exclude all animals in any experimental group that have shown sub-normal appetites when compared with their litter mates receiving the same ration. This would be impossible if the feeding was conducted with several animals in a cage.

Curves 292, 293, 299 and 301 emphasize the point just mentioned. Rats may be reared in the same environment and on the same rations and yet deport themselves differently, depending upon the amount of food that the animal normally desires to ingest. In other words, they will grow in proportion to the natural appetite that they possess.

We have also been able to corroborate the finding of Steenbock with reference to the storage of vitamin B. It is quite evident that the rat is able to store sufficient vitamin in the tissues to carry the animal over several months, provided a large proportion of the feces are eaten. In other words it would seem that the stimulatory substance can be stored, utilized, voided, re-ingested, and the cycle continued for a considerable time, indicating that the demand for vitamin B is urgent but that the quantity needed is not large. On the other hand, if rats are placed on screens for two weeks and then the screen removed, allowing the rat to commence feces eating at that point, the feces seem to have lost their stimulatory properties. This would indicate that the rat does not possess the power to store the vitamin for long periods.

CONCLUSIONS.

Rats are prone, if they have the opportunity, to consume varying quantities of excreta, the ingestion of which seems to keep up a sufficient supply of some stimulatory material (probably vitamin B) to vitiate certain types of experimental work. Just how serious the work of the past has been affected it is difficult to tell. Certainly it will be necessary to repeat certain types of work.

The importance of food intake records for individual animals has been emphasized.

Evidence is cited to show that the rat is limited in its ability to store vitamin B.

[Reprinted from Industrial and Engineering Chemistry,
Vol. 16, No. 2, page 126. February, 1924.]

An Inexpensive Laboratory Mill[1,2]

Designed for the Grinding of Dried Fruits and Similar Materials in the Presence of a Liquid

By R. Adams Dutcher and John F. Laudig

PENNSYLVANIA STATE COLLEGE, STATE COLLEGE, PA.

THE mill described herein is the result of experiments to design a machine for grinding raisins, dried fruit, and other similar viscous materials in the presence of water or other solvent. The apparatus was originally designed to assist buyers of dried grapes in ascertaining the sugar content of the product they were buying, but the writers believe that the mill could be used in a variety of ways in most laboratories, especially in the preparation of colloidal materials and in the grinding of many agricutural, biological, and pharmaceutical materials. This mill was constructed in the college shops at a cost of about $30.00.

In order to obtain the information necessary for properly classifying the dried fruit, the sugar content must be estimated while the shipment is being unloaded and graded, which requires about twenty minutes. The method described in this paper gives fairly accurate results on the determination of the sugar content of dried raisins in a very short time, even in the hands of a nontechnical worker.

DESIGN

In designing the mill shown in Figs. 1 to 4, the following conditions had to be met:

1—Gummy materials had to be ground in the presence of a solvent to remove the sugars as rapidly as possible from the tissues.

2—The material had to be ground to a very fine state of subdivision to insure complete solution of the sugars present.

3—The mill had to be so constructed that no material could be lost during the grinding process.

4—It was also essential that the mill be easily and quickly dismantled and reassembled.

Fig. 1 shows a cross section of the mill as finally built. It consists primarily of two steel disks $4^1/_2$ inches in diameter, the upper one being stationary while the lower one is rotated

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[1] Presented before the Division of Agricultural and Food Chemistry at the 66th Meeting of the American Chemical Society, Milwaukee, Wis., September 10 to 14, 1923.

[2] Published as Contribution No 5 of the Department of Agricultural Chemistry, Pennsylvania State College.

at a speed of 1200 to 2000 r. p. m. according to conditions. In each disk are cut several channels with their cutting edges so arranged that there is a shearing action between the disks. The channels are deep (about $^1/_{16}$ inch) at the center and practically disappear at the periphery. The surfaces of the disks slant downward away from the center (at an angle of about 5 degrees) so that the larger particles will be drawn down by gravity and pass between the plates. The lower disk is connected to the driving motor by means of a $^1/_4$-inch leather belt.

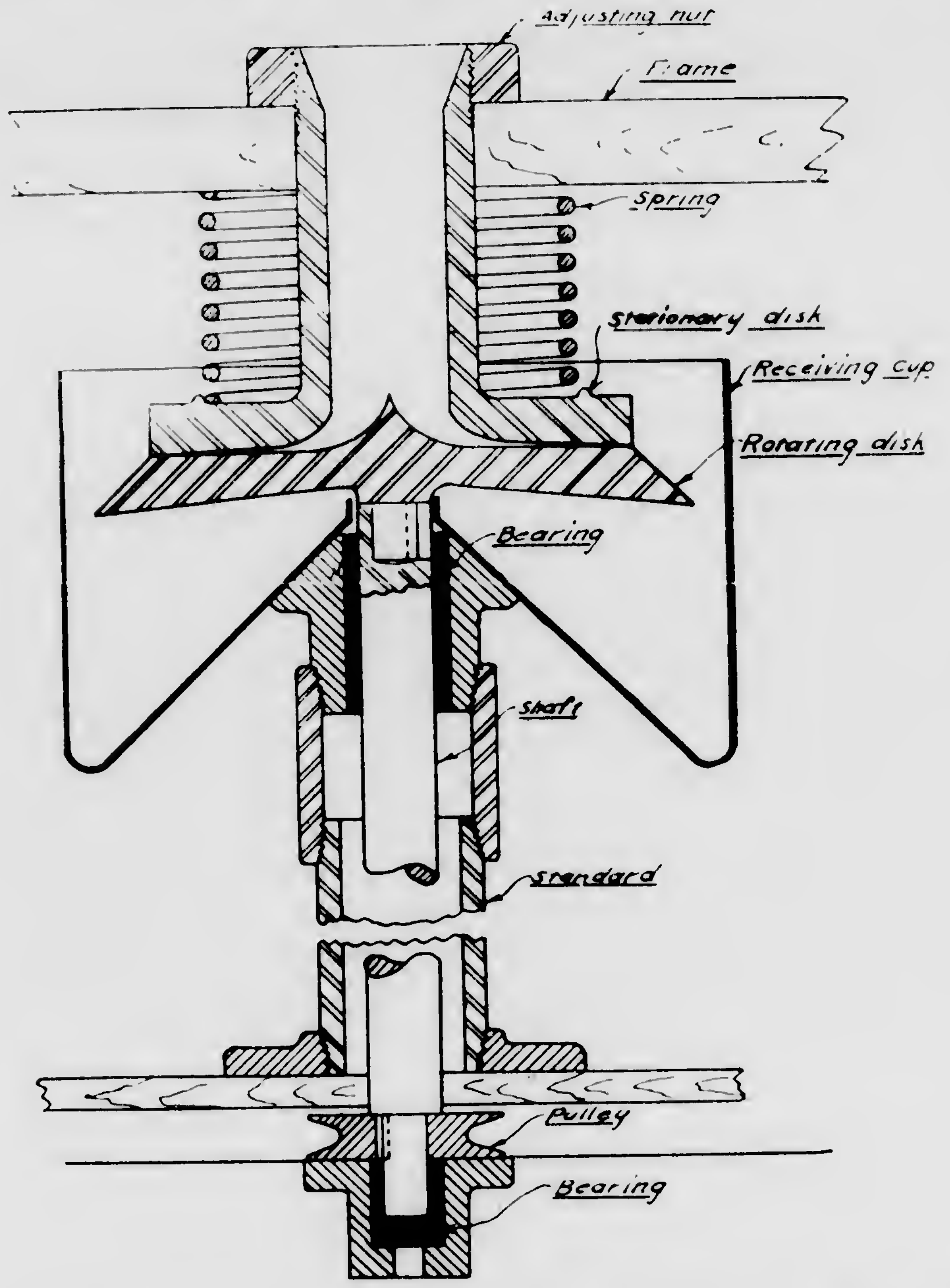

FIG. 1—A CROSS SECTION OF THE MILL SHOWING DISKS, ADJUSTING MECHANISM, RECEIVING CUPS, AND DRIVING SHAFT

The receiving cup is made of tin and is $5^1/_2$ inches in diameter and 5 inches high. The bottom of the cup is conical and made so that it fits snugly over the shaft casing below but not touching the lower disk, as shown in Fig. 1. The interior surface of the cup is perfectly smooth, any crevices being filled with solder. This facilitates cleaning. The receiving cup and lower disk are removable and the upper disk is held in place by a hinged crosspiece on the frame. An adjusting nut at the top of the stationary disk permits regulation of the distance between the plates, and a strong spiral spring imparts the necessary pressure to the grinding surfaces. The framework is constructed of wood 2 inches thick, except for the hinged member carrying the upper disk, which is $1^1/_2$ inches thick. When assembled the mill and frame are $17^1/_4$ inches thick and 25 inches long. (Figs. 2, 3, and 4.) The upper disk is screwed on to a piece of 1-inch pipe, which provides means for both mounting the disk and charging the mill.

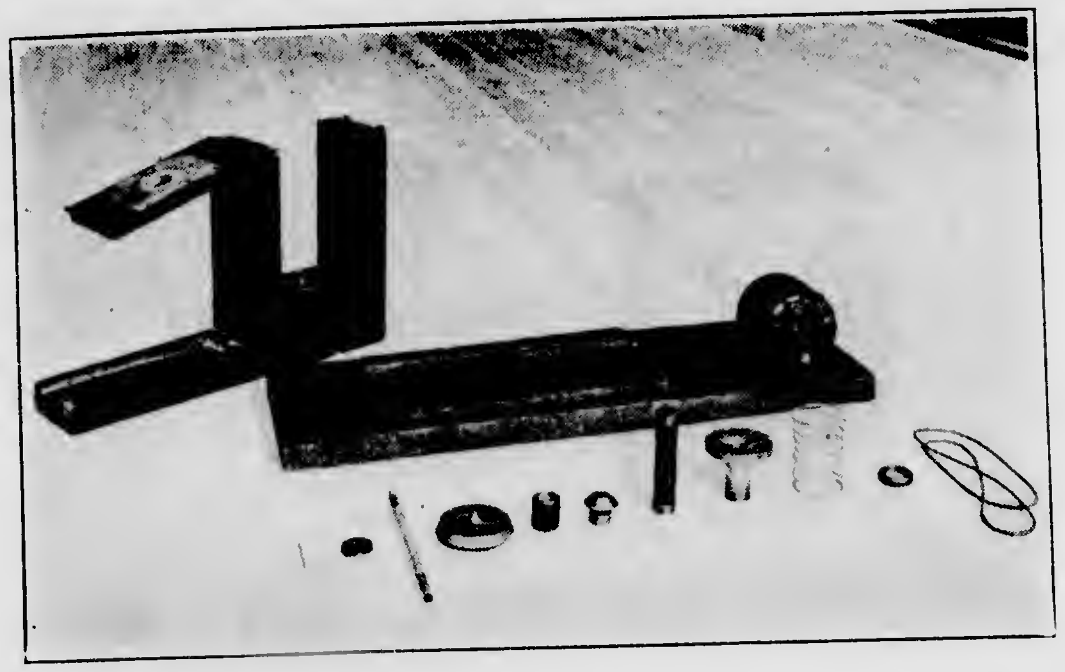

FIG. 2—THE MILL DISMANTLED SHOWING ALL PARTS EXCEPT THE RECEIVING CUP

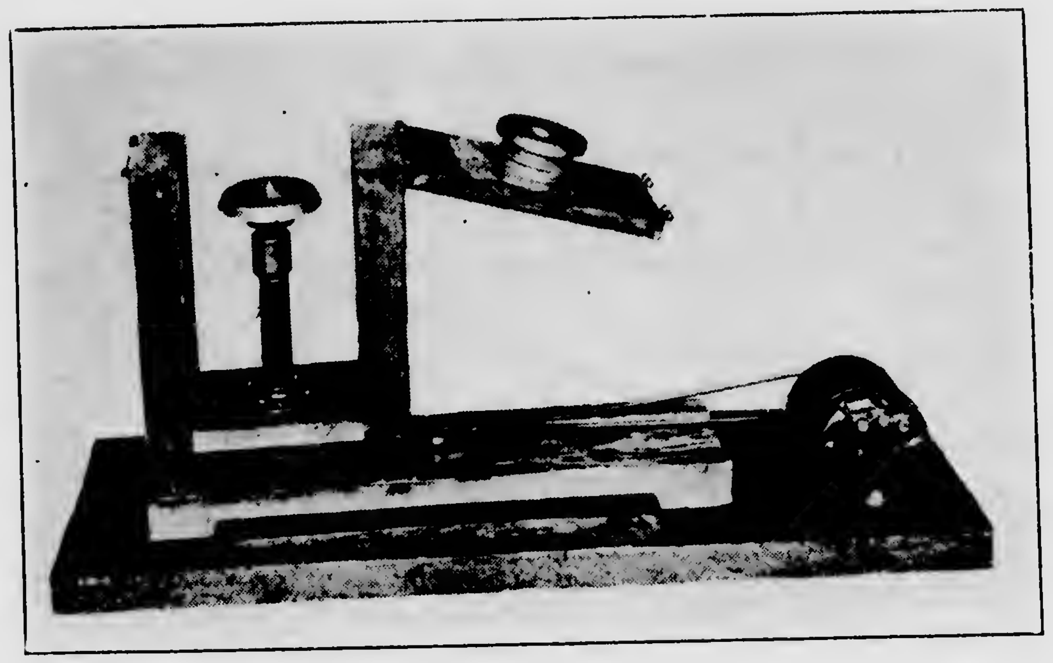

FIG. 3—THE MILL OPEN FOR CLEANING

The finely ground material is whirled from the rotor to the receiving cup, from which it can be drawn off through the drain pipe to a beaker or flask. If the particles in the original material are large, it is advisable to adjust the disks some distance apart at first and pass the suspension through several times, bringing the disks closer together after each pass. By this treatment a very fine suspension can be obtained in a surprisingly short time. The resulting suspension can then be subjected to any analytical procedure desired.

Determination of Raisin Sugars

To insure uniform sampling and facilitate grinding, about 200 grams of raisins are passed through an ordinary food chopper. A 50-gram sample of the ground material is suspended in about 200 cc. of boiling water and passed through the mill as many times as necessary. In the case of raisins three times will usually suffice.

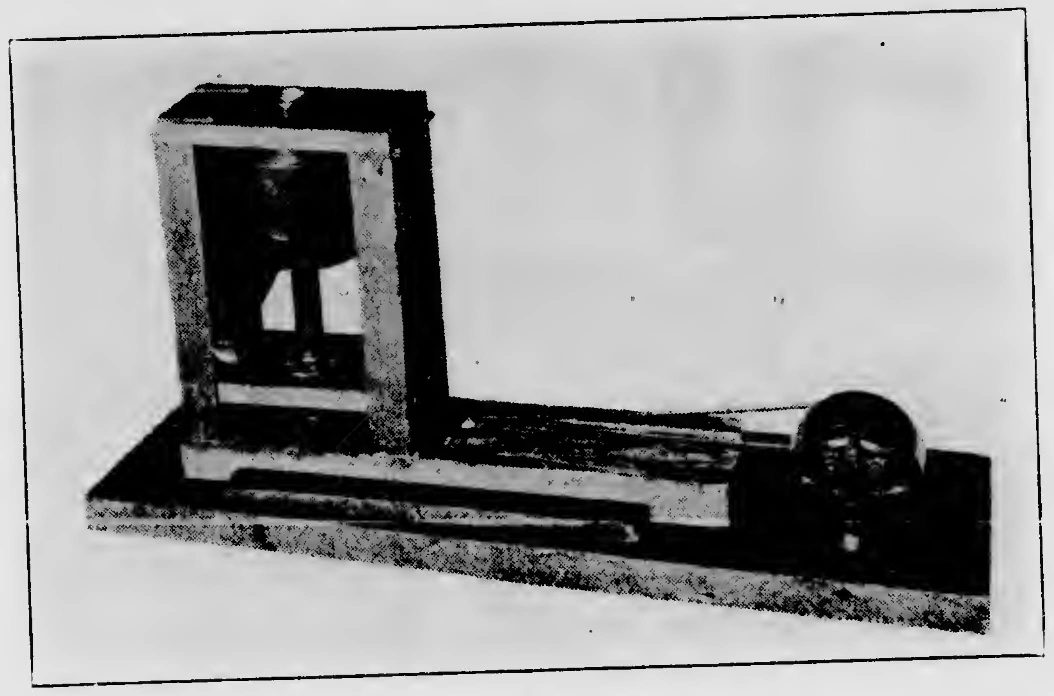

Fig 4—The Mill Complete and Ready for Grinding

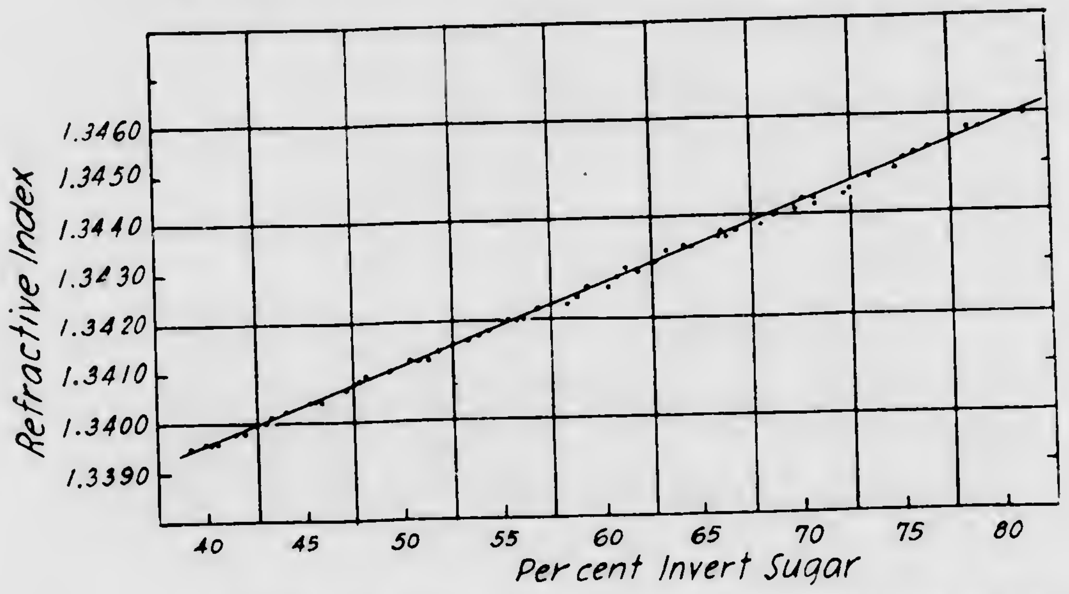

Chart 1—Relation of Refractive Index to Concentration of Reducing Sugars in Raisin Solutions Prepared by the Colloid Mill

The disks and receiving cup are then washed with successive additions of warm water while the mill is running, until all materials are washed into the receiving beaker or flask. The suspension and washings are cooled in running water and made up to 500 cc. volume at 20° C. If analyses are not to be made immediately, four or five drops of toluene should be added to prevent the development of molds.

A few drops of the raisin suspension are then placed between the prisms of an Abbé refractometer and the refractive index of the solution is read. By the use of Chart 1 it is possible to read the percentage of reducing sugars present with a reasonable degree of accuracy. Determinations can be made by this method in less than 20 minutes.

Acknowledgment

The writers wish to acknowledge the invaluable assistance of L. J. Bradford in designing and constructing the mill.

[Reprinted from the Journal of the American Chemical Society,
Vol. XLVI, No. 7. July, 1924.]

[Contribution from the Agricultural Chemistry Laboratories of the
Pennsylvania State College Agricultural Experiment Station, No. 9]

THE COLORIMETRIC DETERMINATION OF CARBOHYDRATES IN PLANTS BY THE PICRIC ACID REDUCTION METHOD

I. THE ESTIMATION OF REDUCING SUGARS AND SUCROSE[1]

By Walter Thomas and R. Adams Dutcher

Received March 29, 1924 Published July 7, 1924

A study of the estimation of sugars in the complex mixtures in plants was commenced by Brown and Morris,[2] followed by the work of Parker[3] and the more complete studies of the Rothamsted group.[4] These workers have tested many points of procedure of the analytical details in the cupric reduction methods and it is apparent that the difficulties have been clearly recognized. Even such skilled and careful workers as Brown and Morris considered the degree of accuracy as being equivalent to 1 mg. of the cupric oxide weighed, corresponding to 0.5 mg. of glucose.

One of the chief objections to copper reduction methods is the difficulty in securing absolute uniformity in the conditions of reduction, which is so essential, since Nef[5] has shown that a number of products, variable in nature and quantity with the concentration of the sugar solution and alkali are formed when the sugar molecule is acted upon by alkaline Fehling solution. The main sources of error due to (a) lack of temperature control and (b) auto-oxidation have been largely overcome by Quisumbing and Thomas;[6] notwithstanding which, considerable difficulty was experienced in our Laboratories in applying it to the determination of small quantities of sugar in leaves and spurs of apple trees, in connection with biochemical studies relating to the effect of fertilizers on the physiological functions of the apple.[7]

A combination of the gravimetric and volumetric methods (Bertrand) is quite extensively used at the present time in biochemical laboratories. The procedure introduces the errors of both; moreover, it is not generally recognized that the standard permanganate solution must be standardized against the cuprous oxide produced by a known quantity of pure glucose and not against oxalic acid. Results obtained in our Laboratories indicate that the values are from 8 to 20% too low when oxalic acid is used.

Inasmuch as colorimetric methods for the determination of sugars have been used with a high degree of accuracy in the sphere of animal physiological chemistry, the present paper is the result of an investigation in the application of Benedict and Osterberg's method[8] to the plants used in the investigation to which reference has been made, due consideration being given to the limitations set by them and to the findings of Falk and Noyes.[9]

General Considerations as to the Applicability of the Method

Of the sugars occurring in plants uncombined, glucose, fructose, sucrose and possibly the pentoses, arabinose and xylose, are the only ones which are important from the standpoint of the plant biochemist. With the exception of sucrose, these have a reducing action on Fehling's copper solution and are accordingly classed as "reducing sugars." These same sugars also have a reducing action on picric acid in alkaline solution, reducing it to a colored substance, presumably picramic acid.

From the experimental data to be presented, it has been found that sucrose also (up to 0.1 *M* concentration) has no reducing action on Benedict and Osterberg's picrate-picric solution. Experiments were also carried out on the quantitative relation between the cupric and picric reducing values of each of these sugars. Inasmuch as these values do not differ very greatly for the respective sugars, we should expect the values obtained by the cupric and picric acid reduction methods to agree with one another within the limits of experimental error.

It was from a consideration of these facts that the application of the colorimetric method was first investigated through the use of lead acetate with the view of simplifying the technique. However, as will be shown, the use of this reagent was not a success.

It will be noticed that maltose has not been mentioned here as being among the sugars occurring in plants, for it is improbable that it occurs except as a laboratory product in the degradation of starch. It is true that Brown and Morris[2] found it in leaves of Tropoelum, but Davis and Sawyer[10] have attributed its presence to the failure of Brown and Morris to inactivate the diastase present, owing to the procedure adopted. The leaves were dried at 50°. Under such conditions considerable quantities of glucose and maltose would be formed and the latter would persist, owing to the destruction of the maltase at the temperature used.

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[1] Read at the Milwaukee meeting of the American Chemical Society, September, 1923.

The cost of this investigation has largely been defrayed by a grant from the Adams Fund for Agricultural Research.

[2] Brown and Morris, *J. Chem. Soc.*, **63,** 604 (1893).

[3] Parker, *Biochem. J.*, **6,** 1 (1912).

[4] Davis, *J. Agr. Sci.*, **5,** 434 (1912–13). Davis and Daish, *ibid.*, **6,** 152 (1914). Daish, *ibid.*, **6,** 255 (1914). Davis and Sawyer, *ibid.*, **6,** 406 (1914). Davis, Daish and Sawyer, *ibid.*, **7,** 255 (1915–16). Davis and Sawyer, *ibid.*, **7,** 352 (1915–16). Davis, *ibid.*, **7,** 737 (1915–16).

[5] Nef, *Ann.*, **357,** 214 (1907); **376,** 1 (1910); **403,** 204 (1914).

[6] Quisumbing and Thomas, This Journal, **43,** 1503 (1921).

[7] Thomas, *Soil Sci.*, **15,** 1 (1923).

[8] Benedict and Osterberg, *J. Biol. Chem.*, **34,** 195 (1918).

[9] Falk and Noyes, *ibid.*, **52,** 109 (1920).

[10] Davis and Sawyer, *J. Agr. Sci.*, **5,** 454 (1912–13).

Following the method of Mulliken[11] and also that of Sherman and Williams[12] no maltose was found in any of the extracts concerned in the present investigation.

Experimental Part

For the convenience of workers who may desire to use this colorimetric method the preparation of the reagents—(a) the picrate-picric solution as developed by Benedict and Osterberg[13] and (b) the mercuric nitrate solution used by them is given here.

Preparation of the Picrate-Picric Solution

Thirty-six g. of picric acid (dried at 60° and purified if necessary)[14] is added to 500 cc. of a 1% solution of sodium hydroxide in a liter flask. This is followed by 400 cc. of hot water. The mixture is shaken occasionally until the picric acid is dissolved, and afterwards cooled and diluted to one liter.

Preparation of the Mercuric Nitrate Solution Used as a Clarifying Agent

A more dilute solution of the mercuric nitrate than that used by Benedict and Osterberg was employed. A solution of 110 g. of mercuric oxide added gradually to 80 cc. of concd. nitric acid is stirred, then heated to boiling, and cooled, and to it is added 30 cc. of a 5% solution of sodium hydroxide. This is then made up to one liter and stored in the dark.

The Standard Glucose Solution

Benedict and Osterberg describe the preparation of a permanent standard.[15] However, thus far, no attempt has been made in the course of this investigation to prepare a permanent color standard. In all our experiments the color of the standard and unknown were developed under exactly the same conditions. Although for low concentrations of sugars a standard containing 0.016% of glucose, which was used in the preliminary experiments, is satisfactory; for general work a standard containing 0.025% of glucose is recommended.

The Picrate-Picric Reducing Power of Fructose, Arabinose and Xylose

The following data are given as indicative of the purity of the sugars employed.

Glucose.—Bureau of Standards product; $[\alpha]^{20}_{D}$, + 52.76; ash, 0.003%; moisture, 0.03%.

Sucrose.—Bureau of Standards product; $[\alpha]^{20°}_{589.25}$, 66.529; ash, 0.003%; moisture, 0.003%.

Fructose.—Pfanstiehl brand; $[\alpha]^{20}_{D}$, —91.66; ash, 0.095%; moisture (after drying under reduced pressure), 0.005%.

l-Arabinose.—Pfanstiehl brand; $[\alpha]^{20}_{D}$, +104.5; moisture, 0.10%.

l-Xylose.—Pfanstiehl brand; $[\alpha]^{20}_{D}$, +18.5; moisture, 0.10%.

The picrate-picric reducing power, by which is meant the ratio between the amounts of glucose and the respective sugar, required to reduce equal amounts of picric to picramic acid, was determined by comparing, in a series of experiments, the color values developed under the same conditions, of equal concentrations of the standard glucose and the sugar, the reducing power of which is to be obtained. Table I gives the comparison of the cupric and picrate-picric reducing powers.

TABLE I

COMPARISON OF THE PICRATE-PICRIC AND CUPRIC REDUCING POWERS OF THE PRINCIPAL SUGARS OF PLANTS

Material	Glucose	Fructose	*l*-Arabinose	*l*-Xylose	Sucrose
Picrate-picric	1.00	0.99	1.01	1.08	0.00
Cupric	1.00	.91	1.00	1.01	.00

Glucose, fructose and sucrose are the sugars that occur in largest amounts in plants. Although the ratio of the picrate-picric reducing power to the cupric reducing power is 99:91, it is improbable that the ratio of glucose to fructose in the plant will be less than unity, for from the work of Davis[16] it seems justifiable to assume that glucose and fructose exist in the leaves and stalks of plants as invert sugar and that they travel to the root in nearly equal proportions (depending upon the consumption in respiratory processes as functions of growth and new tissue formation). For this reason we would expect the results obtained by the two methods to agree closely.

The Failure of Lead Acetate as a Clarifying Agent

The use of this reagent for the removal of proteins, gums, etc., resulted in higher values for reducing sugars by the colorimetric than by the gravimetric method. The disparity, which was far greater than would be accounted for by any differences in the cupric and picric reducing values of the sugars present, was definitely established as being due to the presence of acid-hydrolyzable material (not of pentosan nature),[17] which the lead acetate had not precipitated. An attempt was made to determine the nature of this material by treating 200 g. of leaves according to the method of Schulze and Winterstein.[18] After the decomposition of the mercuric nitrate precipitate, filtration and evaporation of the filtrate under reduced pressure, a thick, dark brown, viscous material was obtained, which thus far has resisted all attempts at crystallization. It showed a marked difference in the cupric and picrate-picric reducing values, the latter being considerably greater than the former. Resort was then made to Benedict and Osterberg's mercuric nitrate solution to precipitate interfering materials, with very satisfactory results.

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[11] Mulliken, "Identification of Pure Organic Compounds," John Wiley and Sons, **1908,** 1st ed., vol. I, p. 32.

[12] Sherman and Williams, THIS JOURNAL, **28,** 629 (1906).

[13] Ref. 8, p. 198.

[14] Folin and Doisy, *J. Biol. Chem.*, **28,** 349 (1910).

[15] Ref. 8, p. 200.

[16] Davis, *J. Agr. Sci.*, **7,** 327 (1916).

[17] A determination of pentosans gave only 0.0026 g. in 50 cc. of the original alcoholic solution, equivalent to 0.08 mg. in 10 cc. of the deleaded solution used in the colorimetric determination.

[18] Schulze and Winterstein, Abderhalden, "Handbuch der Biochem. Arbeitsmethoden," **1910,** vol. 2, pp. 510–521.

Preparation of the Plant Extracts

A representative sample (about 50 g.) of the plant parts is plunged into 95% boiling alcohol, previously redistilled over potassium hydroxide to remove acids and aldehydes, to which has been added 0.2 g. of calcium carbonate to prevent any hydrolyzing action by native acids. Allowing for the moisture present in the plant parts, the alcohol should be brought to a concentration of 70 to 75% by the addition of water, if necessary. The material can remain under alcohol for any length of time that may be convenient to the investigator, when, after being filtered and washed with 75% alcohol, it is dried in an oven at 70°, and then pulverized in a suitable mill[19] to pass an 80-mesh sieve.

The complete removal of the sugars is effected by extracting the powdered plant in a Soxhlet apparatus by using the alcoholic filtrate until the percolate is colorless.[20] The extract is then transferred to a 250cc. or 500cc. volumetric flask, according to the quantity of sugars present. The concentration of sugars in this extract should preferably be from 0.1 to 1.0%. The starch and other polysaccharides remain in the residue.

The Estimation of Reducing Sugars

A 20cc. to 100cc. aliquot portion containing from 0.025 to 0.150 g. of sugars is evaporated at a low temperature to remove the alcohol, the residue is dissolved with water and washed into a 400cc. beaker with 100 cc. of water. A slight excess of mercuric nitrate solution, of which 10 cc. was sufficient in our experiments, is added to the solution. Then, as directed by Benedict and Osterberg, small quantities of solid sodium bicarbonate are added gradually and the liquid stirred after each addition, until all frothing ceases. At this stage the bicarbonate is added carefully until the solution is alkaline to litmus. It is important to avoid any very large excess of sodium bicarbonate because of the subsequent treatment. This solution is then rapidly filtered into a 250cc. flask, the precipitate washed with a little 5% sodium bicarbonate solution, the solution and wash liquid are made up to the mark with water and shaken.

The concentration of this solution with respect to the sugars present should be within the limits 0.01 to 0.07%. From 30 cc. to 50 cc. of this solution is transferred to a 75cc. test-tube and 0.3 to 0.5 g. of zinc dust added. One drop of concd. hydrochloric acid is then added to reduce the small amount of mercury present in the solution, resulting from the solubility of the basic carbonate formed. Care must be taken in this treatment that sufficient sodium bicarbonate is present to prevent the solution becoming acid after the addition of the hydrochloric acid, as otherwise zinc carbonate will be precipitated in the subsequent treatment. The tube is then stoppered loosely and shaken, after which it is allowed to stand for 15 minutes and the contents finally filtered through a hardened filter paper. From 5 cc. to 10 cc. of the filtrate, tested with ammonium sulfide to ascertain whether all traces of mercury have been removed, is pipetted into a 50cc. Pyrex test-tube and 10 cc. of the picrate-picric solution is then added, followed by 2 cc. of a 25% solution of sodium carbonate. When less than 10 cc. of the sugar solution has been used, water equal to the deficiency is added, making the total volume of the solution 22 cc. A standard color solution is simultaneously prepared, using 10 cc. of standard glucose solution, 10 cc. of the picrate-picric solution and 2 cc. of 25% sodium carbonate solution. The tubes are then plugged with cotton and immersed for 20 minutes in a water-bath maintained at 95°; at the expiration of this time the tubes are removed and cooled to room temperature. The standard is diluted to 35 cc., or 70 cc., according to the quantity of sugar in the unknown, which is also suitably diluted,

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[19] In our Laboratories the plants are first ground in a Seck mill and finally in a horizontal plate mill made by H. Dreefs, Halle, Germany.

[20] In our experiments no trace of any sugars was detected after the percolates became colorless.

and the color comparison made in any of the standard colorimeters according to the individual operator's preference.

As Benedict and Osterberg point out, it is absolutely essential to remove all traces of mercury, for apparently mercuric salts possess a reducing action on sugar solutions.[21]

A comparison of results obtained on apple tissues by the method outlined above with those of the cupric reduction method of Quisumbing and Thomas[6] from reducing values determined on smaller quantities of glucose (1 to 10 mg.) than given in the reducing tables of these investigators, is shown in Table II. It will be seen that more uniform and consistent results can be obtained and in much less time by this colorimetric method.

Table II

Experiments Showing the Comparison of Results Obtained on Plant Tissues by the Gravimetric and Colorimetric Methods

Material	Date March	Gravimetric Mg. of glucose in 50 cc. of solution	Colorimetric Mg. of glucose in 50 cc. of solution
Spurs	8	31.4	27.7
	10	28.9	28.0
	13	30.4	28.2
	15	29.0	27.9
	15	28.1	27.6
Leaves	15	20.2	20.6
	15	20.0	20.6
	18	19.0	20.7
	18	19.6	20.7

The method was further tested by adding various known amounts of glucose and fructose to plant extracts of known sugar content.

Table III shows the recoveries obtained.

Table III

Experiments, Using the Colorimetric Method, on the Recovery of Glucose and Fructose Added to Plant Materials

Material	Mg. of glucose added	Mg. of fructose added	Mg. of reducing sugars present in 50 cc. of solution	Total found Mg.	Recovered Mg.	Calcd. Mg.	%
Spurs	10	none	31.8	41.7	9.9	10	99.00
	none	10	31.8	41.8	10.0	10	100.00
	20	none	31.8	51.9	20.1	20	100.50
	none	20	31.8	51.6	19.8	20	99.00
	10	10	31.8	51.8	20.0	20	100.00
Leaves	15	none	20.5	35.6	15.2	15	101.33
	none	15	20.5	35.4	14.9	15	99.33
	10	10	20.5	55.5	19.8	20	99.00

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[21] Knapp, *Z. anal. Chem.*, **9**, 395 (1870).

The Estimation of Sucrose

It has been pointed out (p. 1663) that sucrose up to 0.1 M concentration was found to have no picrate-picric color value.

The experiments were conducted by (1) a direct comparison of sucrose solutions of various concentrations with the standard, the colors of which were developed under the same conditions, and (2) a comparison of the colors developed, likewise under the same conditions, of known amounts of sucrose added to the standard solution with a standard solution of the same concentration with respect to glucose. The results of a series of experiments indicated that the sucrose gave no color value up to 0.1 M concentration.

Inasmuch, therefore, as the products of hydrolysis of sucrose are equivalent amounts of glucose and fructose, it should be possible to determine accurately the quantity of sucrose present in solution not exceeding 0.1 M concentration by determining the increase in color obtained after inversion.

In order to test this, experiments were carried out by hydrolyzing pure sucrose solutions under the Herzfeld[22] condition of inversion,[23] which consists in adding slowly 5 cc. of hydrochloric acid (d., 1.888), in a 50cc. sugar flask containing a thermometer, in a water-bath heated to about 72–73°. The heating is adjusted so that the solution in the flask reaches 69° in three minutes. This temperature is then maintained for seven minutes longer. The solution is then removed and cooled to room temperature and neutralized with sodium hydroxide solution.

Table IV gives the results obtained.

Table IV

Recovery of Sucrose Solution by the Colorimetric Method

Calcd., 33.7 mg. in 50 cc. of solution

Found, mg. in 50 cc. of soln.	33.0	33.1	33.4	33.7	33.6	33.7
Recovery, %	97.9	98.2	99.1	100.0	99.7	100.0

It is of interest to note that the results obtained are not due to any compensating factors, such as, for instance, the sodium chloride formed by the neutralization of the hydrochloric acid, since in parallel blank experiments using the same quantities of hydrochloric acid and sodium hydroxide no color value due to the presence of sodium chloride formed was found in these experiments. This point is emphasized here because of the apparently contradictory results obtained by different workers. Thus, though Okey[24] found a retardation in the rate of production of the colored substance, there appears to be no indication that there was any error in the color value as finally determined, due to the presence of sodium chloride.

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[22] Herzfeld, *Z. Ver. deut. Zuckerind*, **38,** 699 (1888).

[23] Rose, *J. Biol. Chem.*, **46,** 529 (1921), has described a method of inversion by picric acid.

[24] Okey, *ibid.*, **38,** 33 (1919).

Denise and Rose[25] found an error of 17% for solutions containing molar concentrations of sodium chloride, but whether they found an increase or decrease in the color value has not been made clear.

There is, however, no destruction of glucose by the hydrochloric acid under the conditions of inversion adopted. One of us (W. T.) will show, in the following paper, that some glucose can be destroyed under certain conditions, such as when a solution containing glucose is boiled with even a low concentration of hydrochloric acid, as in the secondary hydrolysis of starch conversion products.

On the basis of the diluted solutions finally taken for the determination the concentration of the sucrose in a plant containing even as high a content of sucrose as 10% would only be about 0.025 M.

Summary

The method of Benedict and Osterberg for the determination of sugars in urine has been adapted, with modifications, to the determination of reducing sugars and sucrose in plant extracts. The details of the determination are given.

This colorimetric method is superior to the gravimetric, volumetric and, of course, optical methods for the determination of small amounts of sugars.

The use of mercuric nitrate as a precipitating agent for the removal of proteins, tannins, amino acids and other interfering substances should give truer values for reducing sugars than lead acetate, since the former removes substances having a slight reducing action on Fehling and a much greater reducing action on the picrate-picric solution. The nature of this material has not as yet been determined.

State College, Pennsylvania

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[25] Denise and Rose, *Proc. Assoc. Exp. Biol. Med.*, **20,** 77 (1922).

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[Contribution from the Agricultural Chemistry Laboratories of the Pennsylvania State College Agricultural Experiment Station, No. 10]

THE COLORIMETRIC DETERMINATION OF CARBOHYDRATES IN PLANTS BY THE PICRIC ACID REDUCTION METHOD
II. THE DETERMINATION OF STARCH AND OTHER "RESERVE" POLYSACCHARIDES[1]

By Walter Thomas

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That the determination of starch presents one of the most difficult problems in analytical chemistry is clearly indicated in the literature of the subject. This is due largely to the variable products of hydrolysis formed under the different conditions of conversion.

Of the three chief modes of attacking the problem the method of conversion by acid hydrolysis, even if the products were definitely known, which is not the case, can be applied, in the case of plants, only to the determination of the approximate "reserve" polysaccharides, because other complexes like the hemi-celluloses would also be hydrolyzed. The method, introduced by O'Sullivan,[2] of the conversion by diastase followed by a secondary hydrolysis with hydrochloric acid of the products (the dextrins, glucose and maltose) and the estimation of the glucose formed either by the gravimetric or optical methods is, apart from the difficulties attending the polarization of small amounts of glucose, open to the objection that the accuracy may be affected by the destruction of the sugar by the hydrochloric acid, with the formation of levulinic acid and humin substances.[3] Additional data with respect to the destruction of glucose under these conditions will be given in the experimental part of this paper.

The conversion of starch into glucose and maltose by the mixed enzyme taka-diastase—secreted by *Aspergillus oryzoe*—which possesses very strong converting power has been used by Stone and Wright,[4] Croft Hill,[5] and later more successfully by Davis and Daish,[6] though Horton,[7] with the comparatively large amounts of starch used by him, encountered difficulties which he attributed to the persistence of dextrin, but he conceded that the evidence was not clear.

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[1] Read at the Milwaukee meeting of the American Chemical Society, September, 1923.

The cost of this investigation has largely been defrayed by a grant from the Adams Fund for Agricultural Research.

[2] O'Sullivan, *J. Chem. Soc.*, **45**, 1 (1884).

[3] Davis and Daish, *J. Agr. Sci.*, **5**, 454 (1912–13).

[4] Stone and Wright, This Journal, **20**, 639 (1898).

[5] Croft Hill, *Proc. Chem. Soc.*, **240**, 184 (1901).

[6] Davis and Daish, *J. Agr. Sci.*, **6**, 152 (1914).

[7] Horton, *ibid.*, **11**, 240 (1921).

It is also to be observed that Tottingham and Gerhardt[8] claim to have obtained higher results with salivary digestion than with taka-diastase.

Inasmuch as the application of the Benedict-Osterberg method has proved so satisfactory for the determination of sugars (see preceding paper), its application to the determination of starch, using taka-diastase, was investigated. The experimental evidence[6] is strongly in favor of the view that glucose and maltose are the sole products of conversion by taka-diastase, although, as already stated, Horton[7] considered the possibility of small amounts of dextrin persisting. The analytical error involved in this assumption would, however, be very small. Accordingly, the picrate-picric reducing ratio of glucose to maltose was determined and found to be considerably greater than the cupric reducing ratio, which fact is of importance since, as we shall see, no great error is introduced by a slight variation in the proportion of glucose to maltose in the products of conversion.

Experimental Part[9]

The maltose hydrate used for the determination of the picrate-picric reducing power was the Pfanstiehl brand; $[\alpha]_D^{25} = +131.0°$; water, 0.15%; ash, 0.02%.

Ten-cc. portions of solutions containing equal concentrations of the standard glucose and of the maltose hydrate (calculated as the anhydride) were taken, and the color developed, in a series of experiments, under the conditions described in the preceding paper (p. 1664). The value obtained in this way was 0.91 ± 0.01. This compares with a cupric reducing ratio of glucose to maltose of 0.61.

The preliminary experiments with the method were made on potato starch. It gave on analysis: protein (N × 6.25), 0.168%; ash, 0.270%; crude fiber, 0.00%; ether extract, 0.370%; moisture, 12.860%. It was freed from moisture by drying under reduced pressure at 120° for eight hours. The starch determinations were all made on this vacuum-dried product.

The Ratio of Glucose to Maltose

The value of the ratio of glucose to maltose was determined by resolving the equations,

$$g + 0.61\ m = R \tag{1}$$
$$g + 0.91\ m = R' \tag{2}$$

where R and R' represent the sum total of the cupric and picrate-picric reducing powers, respectively, of glucose (g) and maltose (m), as indicated by the amounts of the reduction products obtained from equal volumes of the solution containing the sugars from the diastatic conversion of the starch, by the gravimetric and colorimetric methods, respectively.

In a series of 19 experiments[10] using 0.25 g. of the starch and in eight experiments using 0.1 g. starch, the ratio was found to range from 1.9 to 2.3, with a mean value of 2.05; that is, when about 65% has been converted into glucose. Whether or not this represents the equilibrium position at these low concentrations of the direct and reversible reactions between glucose and maltose has not been determined.

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[8] Tottingham and Gerhardt, *Ind. Eng. Chem.*, **16**, 139 (1924).

[9] For details concerning Benedict and Osterberg's solutions used, the reader is referred to the preceding paper (p. 1664).

[10] These experiments were made using three different lots of the enzyme preparation, one of which was known to be at least a year old, another about three years old. The age of the third lot was not known.

The cupric reducing ratio of glucose to maltose varies from 0.52 to 0.69 according to the conditions of reduction. Since, however, the difference between the picrate-picric reducing power of glucose and maltose is not very great and the ratio of glucose to maltose is approximately equal to 2.0 under the conditions to be described, it is not necessary to obtain the cupric reducing value; for by solving the equations

$$g/m = 2.0 \tag{3}$$

$$g + 0.91\ m = R' \tag{4}$$

we can estimate the starch by a colorimetric determination alone without a great sacrifice of accuracy. An example will make this clear.

In one experiment R' was found to be 0.2618 g. in terms of standard glucose.

Hence $$g + 0.91\ m = 0.2618 \tag{5}$$

and substituting we have $2\ m + 0.91\ m = 0.2618$, whence $m = 0.09$ and $g = 0.18$.

Starch value of g $= 0.18 \times 0.900 = 0.1620$

Starch value of m $= 0.09 \times 0.947 = 0.0852$

Found	0.2472
Calcd.	0.2480

That the error involved by taking the ratio of glucose to maltose as 2.0 is small can be seen by calculating the values obtained in an experiment in which an extreme ratio (2.3) was obtained.

R for the total solution (250 cc.) = 0.2365 g.

R′ = 0.2600 g.

Resolving Equations 1 and 2 we find a value for starch of 0.2443 g., and from Equations 3 and 4 of 0.2454. The difference is 0.0011 g.

The Method

The details of the method for determining starch are as follows.

From 1 to 4 g. (according to the quantity of starch present; the absolute amount of starch should not exceed 0.3 g.), of the vacuum-dried residue[11] freed from all traces of sugars is gelatinized by heating to boiling for 40 minutes on a steam-cup or water-bath with 200 cc. of water with continued stirring for at least ten minutes, in order to prevent the accumulation of starch aggregates, and occasional stirring thereafter. It is then cooled to 38° and incubated at this temperature for 24 hours, with 0.1 g. of the enzyme preparation and 2 cc. of toluene. During the incubation it is desirable to stir the solution gently and frequently during the first few hours since the starch gel tends to separate into two phases on standing. Moreover, the toluene[12] which is lost through evaporation is replenished as needed. At the end of this period the solution is heated to boiling in a steam-cup or water-bath for 15 minutes, in order to render the enzymes inactive, and afterwards the residue is filtered and washed thoroughly by decantation with water into a carefully calibrated 250cc. flask. An aliquot portion (30 cc. is sufficient) is taken, and 5 cc. of the mercuric nitrate reagent and sodium bicarbonate, are added, followed by filtration and treatment with zinc and hydrochloric acid as described in the preceding paper (p. 1666); 1 to 5 cc. is transferred by means of an Ostwald pipet to a 50cc. Pyrex test-tube, diluted to 10 cc., and followed by 10 cc. of the picrate-picric solution and 2 cc. of 25% sodium carbonate solution. The color of this solution and the standard is developed under the same conditions as already described in the preceding paper (p. 1666).

In calculating, the dilution factor for the mercuric nitrate solution added must, of course, be used, and a blank determination on the same amount of the taka-diastase (0.1 g.) used in the conversion experiment is carried out simultaneously. The blank experiments were found to be remarkably uniform, being 66 ± 0.03 mg. for the total amount of enzyme used. The recovery by this method of 0.25 g. of potato starch added to (1) spurs and (2) leaves of apple trees in six experiments was from 98.4 to 99.4% (calcd. 99.2%).

Experiments on a Secondary Hydrolysis with Hydrochloric Acid

A comparison of the results obtained in this manner was made with those from a secondary hydrolysis with hydrochloric acid, since a number of workers[13] have reported losses of glucose amounting to 1.0–1.5% as a result of hydrolysis for two and a half hours with 2.5% actual hydrochloric acid, due apparently to the formation of levulinic acid and humin substances; while others,[14] using the same concentration of acid, have not had the same experience.

From the results of the determinations of "reserve" polysaccharides, to be described later, it is certain that a concentration of 0.5% of the catalyst, acting at the boiling point for four hours, is sufficient for the conversion of the comparatively small quantities of "reserve" polysaccharides used for these colorimetric determinations. The inference from the results given in Table I is that glucose has been destroyed even at this low concentration.

TABLE I

COMPARISON OF THE RESULTS OBTAINED BY HYDROLYSIS WITH (1) TAKA-DIASTASE ALONE AND (2) TAKA-DIASTASE FOLLOWED BY HYDROCHLORIC ACID

Hydrolysis with taka-diastase only		Hydrolysis with taka-diastase followed by HCl	
Starch recovered		Starch recovered	
G.	%	G.	%
0.2475	99.7	0.2430	98.0
...	..	.2430	98.0
.2466	99.4	.2418	97.5
...	..	.2425	97.8
.2475	99.7	.2432	98.1
...	..	.2425	97.8

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[11] See p. 1666 of the preceding paper.

[12] Toluene was first used by Fischer, *Ber.*, **27,** 2985 (1894); **28,** 1429 (1895).

[13] Noyes, Crawford, Jumper, Flory and Arnold, THIS JOURNAL, **26,** 266 (1904). Ref. 3. Ref. 8.

[14] Olmsted, *J. Biol. Chem.*, **41,** 45 (1920). Walton and Coe, *J. Assoc. Official Agr. Chem.*, **6,** 350 (1923).

The Determination of Other "Reserve" Polysaccharides

Because of the important physiological functions that polysaccharides other than starch may exercise as reserve materials in plants, no special difficulties were found in the extension of the colorimetric method to the determination of materials that are, like the hemi-celluloses for example, hydrolyzed by dil. acids and which are utilized by the plant through transformation into the corresponding sugars by elaborating the specific enzyme. However, the results by this means are to be regarded as only relative; moreover, the possible destruction of glucose may vitiate the results to some extent. Since this work has been carried out, Tottingham and Gerhardt[15] have indicated their preference for hydrolysis with 1% sulfuric acid rather than 2.5% hydrochloric acid, because of the smaller quantity of reducing substances precipitated by the clarifying agent from the former hydrolysates. These authors do not, however, appear to have recognized the work of Englis and Tsang,[16] who have shown that the losses of sugar when basic lead acetate is used as a clarifying agent were due in a large measure to the insoluble salts formed by the *deleading* agents which varied considerably in their effect; their results clearly indicate that disodium phosphate is the most satisfactory deleading agent. This is in conformity with unpublished data obtained by the present writer.

The Method

From 0.5 to 1.0 g. of the vacuum-dried residue from which all traces of sugars have been removed, as already stated in the preceding paper (p. 1666), is treated in a Pyrex Erlenmeyer flask with 200 cc. of water containing 0.5% of actual hydrochloric acid and boiled for four hours under a reflux condenser. After cooling, the solution is exactly neutralized with sodium hydroxide and filtered into a calibrated 250cc. volumetric flask. After washing the filter with water, the combined filtrate and wash waters are made up to the mark and shaken.

The subsequent procedure is exactly as described under the determination of starch, using suitable aliquot portions to keep within the concentration limits.

Table II

Material	Actual HCl present in solution %	"Reserve" polysaccharides as Glucose Gravimetric %, dry basis	Colorimetric %, dry basis
Leaves	0.5	12.00	10.20
	0.5	10.60	10.48
	1.0	10.00	10.00
	1.0	10.50	10.05
Seedling spurs	0.5	12.00	12.00
	0.5	12.60	12.05
	1.0	11.90	11.00
	1.0	10.50	11.15
	1.0	10.50	11.00

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[15] Ref. 8, p. 140.

[16] Englis and Tsang, This Journal, **44**, 865 (1922).

A comparison of the cupric and picrate-picric reduction methods in these determinations of "reserve" polysaccharides is given in Table II.

These results would indicate that no advantage is offered in the hydrolysis of this class of material by increasing the percentage of hydrochloric acid over 0.5%, for there is clearly a destruction of glucose even with 1% hydrochloric acid.

Summary

A colorimetric method for the determination of starch by means of conversion with taka-diastase without a secondary hydrolysis has been described. Under the conditions of conversion, the ratio of glucose to maltose is remarkably constant and the analytical error introduced by regarding this ratio as 2.0 is very small, since the picrate-picric reducing ratio of glucose to maltose is relatively high. A method also for the determination of other "reserve" polysaccharides has been given.

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THE DETERMINATION OF NITROGEN IN CONNECTION WITH THE WET COMBUSTION METHOD FOR CARBON.*

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(Received for publication, June 6, 1924.)

In the study of the carbon metabolism of *Fusarium lini* on glucose solutions[1] the wet combustion method of Rogers and Rogers,[2] as modified by Gortner,[3] was found very satisfactory for determining carbon in the mycelium. Since in work of this kind two cultures grown under identical conditions often show great variations in the nature of their growths, it is desirable to make as many determinations as possible on the same sample. In connection with the above mentioned study it was desirable to determine nitrogen as well as carbon in the mycelium. Due to the fact that only a few tenths of a gram of material were available in each case, it occurred to the authors that the residue from the digestion with chromic acid might be used for the determination of nitrogen by simply making alkaline and distilling the ammonia as in the usual Kjeldahl procedure.

To test this method wet combustions were run on asparagine, digesting with 50 cc. of carbon dioxide-free water, 5 gm. of potassium dichromate, and 75 cc. of concentrated sulfuric acid, at a temperature just below the boiling point for about 2½ hours or until aeration was complete. Because of the large amount of acid to neutralize, the residue was divided, approximately equal amounts being placed in two large Kjeldahl flasks, diluted, made alkaline with sodium hydroxide solution, and the ammonia from both flasks distilled into the same standard acid solution. Table I indicates practically theoretical results on asparagine which show that both amide and amino nitrogen may be determined in this manner.

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[1] Anderson, A. K., and Willaman, J. J., *Proc. Soc. Exp. Biol. and Med.*, 1922–23, xx, 108.

[2] Rogers, R. E., and Rogers, W. B., *Am. J. Sc. and Arts*, series 2, 1848, v, 352.

[3] Gortner, R. A., *Soil Sc.*, 1916, ii, 395.

Since the method was to be used on fungus mycelium the next thing to consider was its application to such a material. Since no large amount of *Fusarium lini* mycelium was available a quantity of puffballs, another fungus material, was gathered, dried, and submitted to analysis. As indicated in Table I, lower results were obtained by the wet combustion method than by the Kjeldahl-Gunning method. At first these low results were attributed to incomplete oxidation of the material, consequently several modifications of the procedure were tried, such as varying the time of heating, and the amounts of acid and potassium dichromate used. No modification gave higher returns of nitrogen than the usual wet combustion method. Since the wet combustion method gave uniformly low results for nitrogen it was thought that a factor might be worked out which could be used to convert these results into true nitrogen values as determined by the Kjeldahl method. For this purpose dried *Fusarium lini* mycelium was used. In this case the wet combustion nitrogen values agreed exceedingly well with those obtained by the Kjeldahl method (see Table I). Apparently the puffballs contained some material which was interfering with the nitrogen determination by the wet combustion method. If chlorides were present the reaction represented by the following equation might be taking place, thus accounting for the loss of nitrogen:

$$K_2Cr_2O_7 + 2NH_4Cl = 2KCl + Cr_2O_3 + 4H_2O + N_2$$

A qualitative test for chlorides in the dried puffballs proved to be strongly positive. The *Fusarium lini* mycelium being grown on a chloride-free medium contained no chlorides. To settle this question asparagine was analyzed again in the presence of varying amounts of hydrochloric acid. The results in Table I show plainly a loss of nitrogen. To show the application of this method to other materials, analyses were made of casein and gelatin, both of which were chloride-free. The results agreed well

with those obtained by the Kjeldahl method. The addition of sodium chloride to these materials lowered the nitrogen by the wet combustion method. On the other hand, egg albumin,

TABLE I.

Comparison of Kjeldahl and Wet Combustion Method for Determining Nitrogen.

Method.	Carbon.	Nitrogen.	
		Wet combustion.	Kjeldahl.
Asparagine.			
	per cent	*per cent*	*per cent*
Theoretical	31.98	18.65	18.65
Usual wet combustion	32.19	18.43	18.48
	32.19	18.68	18.61
	31.95	18.66	
" " " + 1 cc. 0.1 N HCl	32.16	17.86	
" " " + 5 " 0.1 N "	32.17	17.30	
" " " + 25 " 0.1 N "	32.28	15.25	
Dried puffballs (chlorides present).			
Usual wet combustion	40.64	7.56	8.62
	41.22	7.42	8.61
	40.54	7.42	8.64
Aeration method	40.82	8.54	8.58
	40.76	8.56	8.62
Dried *Fusarium lini* mycelium (chloride-free).			
Usual wet combustion	46.26	3.46	3.44
	46.76	3.54	3.48
Casein (chloride-free).			
Usual wet combustion	50.81	12.37	12.25
	50.77	12.29	12.35
	50.72	12.44	12.31
" " " + 0.1 gm. NaCl	50.72	7.84	
	50.79	8.23	
Aeration method in presence of 0.1 gm. NaCl	50.87	12.48	
	50.61	12.49	
	50.72	12.45	

TABLE I—*Concluded.*

Method.	Carbon.	Nitrogen.	
		Wet combustion.	Kjeldahl.
Gelatin (chloride-free).			
	per cent	*per cent*	*per cent*
Usual wet combustion	42.54	15.63	15.59
	42.78	15.80	15.75
	42.75	15.86	15.70
" " " + 0.1 gm. NaCl	42.71	8.56	
	42.65	9.01	
Aeration method in presence of 0.1 gm. NaCl	42.43	15.77	
	42.67	15.84	
Egg albumin (chlorides present).			
Usual wet combustion	43.82	11.82	12.86
	43.70	11.66	12.82
Aeration method	43.91	12.49	12.53
	43.84	12.45	12.37
	43.75	12.69	

which contained chlorides, gave lower results by the wet combustion method than by the Kjeldahl method, showing similar results to those obtained on puffballs.

It is evident from the above that chlorides interfere with the determination of nitrogen by the wet combustion method. Therefore, in order to make the method of value for biological materials a simple method must be devised for removing chlorides. Two methods were tried, one of which proved satisfactory. The unsuccessful attempt was made by conducting the oxidation in the presence of silver sulfate. The results were no higher than when no silver was present. The successful method consisted of an aeration of the sample in the presence of concentrated sulfuric acid before the addition of the potassium dichromate. The liberated hydrochloric acid was absorbed in a U-tube containing a saturated solution of silver sulfate. The sample was placed in the digestion flask, 50 cc. of concentrated sulfuric acid were added, aspiration was started, and the mixture heated to just below the boiling point. The time of aeration depends upon the amount of chlorides present, for the average sample a period of 30 minutes is sufficient. With a 0.2 gm. sample to which 0.1 gm. of NaCl had been added, the chlorides were completely removed in 45 minutes. Heating is necessary for the rapid removal of chlorides, the charring which occurs does not interfere with the carbon determination because the apparatus remains intact during the entire procedure. Without heating all the chlorides could not be removed from a sample containing 0.1 gm. of NaCl in 6 hours. After the aeration was completed, 75 cc. of a solution containing 5 gm. of potassium dichromate dissolved in 25 cc. of concentrated sulfuric acid and 50 cc. of carbon dioxide-free water were placed in the separatory funnel and slowly run into the combustion flask. By this modification there was no danger of loss of carbon dioxide by opening the flask to add solid potassium dichromate. The determination was then continued as in the usual wet combustion method.

An examination of Table I indicates that the aeration method is very satisfactory for removing the interfering chlorides. The nitrogen values obtained on puffballs and egg albumin, which contain chlorides, after aeration, correspond with those obtained by the Kjeldahl method. Sodium chloride added to casein and gelatin may be removed by aeration and the results agree with those obtained on the chloride-free material. It should also be noted that the treatment given the sample in the aeration method does not alter the carbon values.

The writers feel that the wet combustion method for determining nitrogen will prove of value where it is desirable to determine both nitrogen and carbon on the same sample. It is believed that the method will give good results on any material to which the Kjeldahl method is applicable. When chlorides are present it is necessary to aerate the sample in the presence of hot sulfuric acid before adding the potassium dichromate.

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DRIED YEAST PRODUCT AS A SUPPLEMENT TO A GOOD POULTRY LAYING RATION AS A MEANS OF INCREASING EGG PRODUCTION

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DRIED YEAST PRODUCT AS A SUPPLEMENT TO A GOOD POULTRY LAYING RATION AS A MEANS OF INCREASING EGG PRODUCTION

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Previous work[1, 2, 3, 4] has shown that diets deficient in vitamin B produce marked changes in many of the vital organs of fowls, especially the reproductive organs.

With these facts in mind it was considered of practical and scientific importance to know whether or not a good poultry ration, such as is advocated by leading poultrymen, could be supplemented to advantage by the addition of some substance especially rich in vitamin B.

Due to the unusual publicity given to yeast preparations during the past two years, this Station has been receiving a large number of requests for information concerning the advisability of using yeast preparations in poultry feeds. A number of such feeds are already on the market and are being advertised as valuable feeds for the stimulation of increased egg production. As far as we can find by the examination of the literature, no experimental evidence is available to substantiate these claims. Lee[5] has made the observation that yeast was of little value in the feeding of laying hens. It is reasonable to expect that a poor poultry ration could be supplemented advantageously by the addition of dried yeast but inasmuch as intelligent poultrymen are feeding the accepted poultry rations advocated by the various experiment stations of the country, the problem which really needs solution is—"Can yeast be used to advantage in supplementing the better type of poultry ration? In other words will an excess of vitamin B, over that ordinarily contained in the better poultry rations, stimulate better appetite, better growth, higher egg production and also influence the size and weight of eggs?"

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[1] Funk, C. and Douglas, M.—J. Physiol. 47, 475. 1914.

[2] McCarrison, Robt.—Ind. J. Med. Res. 6, 550. 1919.

[3] Dutcher, R. A. and Wilkins, S. D.—Am. J. Physiol. 56, 437. 1921.

[4] Souba, A. J.—Am. J. Physiol. 64, 181. 1923.

[5] Lee, A. R.—J. Am. Assoc. Instr. and Invest. Poultry Husbandry 7, No. 10, pp. 74–76. 1921.

The experiments described in this paper were initiated in November, 1921, at the Pennsylvania State College Experiment Station in cooperation with a prominent yeast manufacturing concern.[*] The yeast used in these experiments was dried yeast known as Yeast Foam.

PLAN OF THE EXPERIMENT

In general, the plan of the experiment was to feed a large number of pullets and hens a satisfactory poultry ration consisting of mash, scratch feed, grit and oyster shell, supplementing certain experimental groups with varying amounts of the yeast product. Data were to be collected on the influence of such factors as (1) yeast product *vs.* no yeast product, (2) dry mash *vs.* wet mash with and without yeast product added, and (3) electric lights to increase the length of the hen's working day *vs.* no electric lights, with and without the yeast product.

Data were to be collected with regard to (1) body weights of individual birds, (2) the actual number of eggs produced per bird by use of trap nests, (3) the weight of eggs produced per bird, (4) size of eggs produced, and (5) food intake per bird.

THE RATIONS

At the beginning of the experiment each pen contained 45 females. Pens 1 to 8 inclusive consisted of pullets, while pens 9 and 10 consisted of yearling hens. All pens received the same standard scratch grain mixture of 3 lbs. of cracked corn and 2 lbs. of wheat. In all the pens this material was fed separately from the mash.

Pen 1 receeived a dry mash which consisted of equal parts of wheat bran, wheat middlings, oat chop, corn meal and meat scraps and 25 per cent yeast. Electric lights were used in the morning only.

Pen 2 was fed a dry mash of equal parts of wheat bran, wheat

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[*] A part of the expense of this investigation was defrayed by a grant from the Northwestern Yeast Company, Chicago, Illinois.

middlings, oat chop, corn meal and meat scraps. Electric lights were used in the morning only.

Pen 3 received the same ration as Pen 1. This pen, however, did not receive artificial light.

Pen 4 was fed a dry mash ration consisting of equal parts of corn meal, wheat bran, wheat middlings, oat chop, meat scrap—with 15 per cent yeast. Electric lights were not used.

Pen 5 received the same ration as Pen 2. Electric lights were not used.

Pen 6 received the same ration as Pen 1 but was fed a moist mash. No artificial light was used in this pen.

Pen 7. The mash ration for this pen was similar to that of Pen 4 except the mash was fed moist. No artificial light was used in this pen.

Pen 8. This ration was identical with that of Pen 5 with the one exception that it was moistened. No artificial light was used in this pen.

Pen 9 received the same ration as Pen 3. It will be remembered that this pen contained yearling hens.

Pen 10 was fed the same ration as Pen 5. This pen also contained yearling hens.

THE EXPERIMENTAL GROUPS

In order that the usual criticism regarding the small number of individuals per pen might not be applied to this work it was decided that all pens should consist of at least 45 birds. It was desirable that these numbers be increased to 100 birds per pen but this was impossible owing to lack of space. All of this work was done with pullets with the exception of two pens of yearling hens in order that previous dietary history should not be an influencing factor in their second laying season.

The experimental pens were arranged according to the scheme outlined in Table I.

The three hundred sixty (360) White Leghorn pullets selected for this experiment were taken from the regular flock of pullets raised on the poultry farm of the Pennsylvania State College. The birds were not pedigreed although pedigreed males have been used in the breeding pens for the past six years. The pullets used in this experiment were of various ages.

TABLE I.—THE EXPERIMENTAL PENS

Pen	No. of Birds	Yeast Product	Mash	Electric Lights
		PULLETS		
1	45	25%	dry	lights
2	45	none	dry	lights
3	45	25%	dry	none
4	45	15%	dry	none
5	45	none	dry	none
6	45	25%	wet	none
7	45	15%	wet	none
8	45	none	wet	none
		HENS		
9	45	25%	dry	none
10	45	none	dry	none

Great care was taken, however, to have each pen uniform with regard to quality, age and size of birds in order that the results obtained might be comparable. In other words, each flock of pullets contained some early hatched, some medium hatched and some late hatched birds. Many of the early hatched pullets in each pen molted in the early fall. The late hatched birds were not fully developed and, therefore, their laying period did not begin until late winter.

In the case of the hens used in the experiment, conditions were different. These birds were retained as having been good producers during their pullet year and both pens were uniform as to the individuals in the pens as well as the pens themselves.

FEEDING

All pens were watered and fed scratch grain early in the morning and as late in the afternoon as possible. During the winter months they were also fed a small amount of scratch grain mixture in the early afternoon. This was for the purpose of increasing the amount of exercise. Yeast was fed during the middle of the forenoon. The pullets in pens 1 and 2 received about 13 hours of light per day. In the late fall and early winter this necessitated turning the lights on about 4:30 in the morning and again for a little time in the evening. No artificial light was given to the birds after 5 P. M. of each day.

The food (both grain and mash) was weighed back each week and the amount recorded. An accurate account was kept of the grit and oyster shell consumed by each pen.

Each bird was weighed on Monday night of each week, and the data recorded. All eggs were collected at noon and again at night. These eggs were weighed and measured daily. The birds were weighed in ounces, while the eggs were weighed in grams and measured in millimeters.

All birds were trapnested. These nests were inspected frequently, in order that the birds might not be compelled to remain away from their feed any longer than necessary.

Colds, roup, canker and chickenpox developed in all pens. Every effort was made to eliminate and control these diseases. Each bird was treated individually, yet birds in all pens suffered to some extent. Pens 3 and 6 suffered most.

DISCUSSION

1. *The influence of feeding a high level of yeast with and without electric lights to birds receieving a dry mash ration.*

Pen 1 (receiving electric lights and 25 per cent yeast) was superior to pen 2 (receiving electric lights and no yeast) in every respect. Significant differences were quite evident with regard to body weight, egg production and size and weight of eggs. It should be noted also that the food intake was somewhat greater in pen 1, amounting to 5.50 lbs., more per bird for the ten month period than was observed in pen 2. This is in harmony with the findings of other investitgators[6, 7] with regard to the appetite provoking properties of vitamin B. Pen 1 also exceeded pen 2 in egg production to the extent of 12.57 eggs per bird or slightly over one dozen eggs per fowl for the entire experimental period, and also showed a significant difference of 3.010 ± 0.632 ounces in weight of eggs per bird per month or 1.875 pounds of eggs for the entire experimental period. Under this forced egg production with electric lights the birds of pen 1 also maintained their body weights better, there being a significant difference of 2.23 ± 0.438 ounces per bird per month.

The birds in pen 2 were laying more eggs than those in pen 1 during the first few weeks of the experiment and the coefficient of variation was smaller, which would indicate that the birds in pen 2 were laying more uniformly during that time.

For these reasons it seems plausible to conclude that the yeast product must have stimulated metabolic processes resulting in a greater appetite, greater body weieght and a larger number of eggs of larger size and weight.

2. *What part did the electric lights play?*

In this comparison of groups we have pen 2 which was discussed above and pen 5 which was not subjected to lights. No yeast was fed to either of these groups. The results seem to indicate quite clearly that the lights had but one beneficial effect (that of a more even distribution of egg yield), the groups deporting themselves quite similarly in other respects. This would tend to show, therefore, that the superiority of pen 1 over pen 2 was not due to electric lights. Our observations that electric lights had little effect, other than to change the distribution of eggs produced, is in harmony with the findings of other investigators.[8]

The same conclusions can be drawn in the case where yeast was fed with and without electric lights. A comparison of pen 1 with pen 3 shows no significant differences in favor of the lighted pen, when the data are figured on the ten month basis with the possible exception that the number and weight of eggs laid per bird seem to approach significance. More eggs, however, were laid by the birds of pen 1 when egg prices were high, namely, in the late fall and winter, than were produced by the birds of pen 3. However, electric lights were responsible for a more even distribution of egg production throughout the period.

3. *Comparison of yeast at different levels.*

The results obtained in pens 3 and 4, where yeast was fed at levels approximating 25 and 15 per cent of the ration can be compared with pen 3 which received no yeast. All groups were fed dry mash and were not subjected to electric lights.

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[6] Karr, W. C.—Proc. Soc. Expt. Biol. Med. 17, 84. 1920.

[7] Sherman, H. C.—The Vitamines, p. 92, Chemical Catalogue Co. 1922.

[8] (a) Rice, J. E.—The Use of Artificial Light to Increase Winter Egg Production, pp. 10, 11, 14, 15, and 16. Reliable Poultry Journal, Dayton, Ohio. 1921.

(b) Lewis, H. R.—Hints to Poultrymen. N. J. Expt. Station Circular, Vol. 9, No. 1. 1920.

In comparing pen 3 with pen 4 we find that pen 3 consumed more food. In view of the fact that pen 4 showed a significant increase in egg production and weight of eggs, we are led to conclude that the difference is due to the superior physical condition of the birds in pen 4 rather than to any undesirable effect due to the larger amount of yeast ingested by pen 3.

When pen 3 (25 per cent yeast) is compared with pen 5 (no yeast) the results are more conclusive. While no difference was noted in egg production, pen 3 produced somewhat larger and heavier eggs and ate more food.

A comparison of pen 4 (15 per cent yeast) and pen 5 (no yeast) is somewhat surprising in view of the results obtained in pens 1 and 2 for the reason that no significant differences were evident in food intake. Significant differences are apparent, however, with regard to egg production, weight of eggs and body weight of birds in favor of the yeast fed pen.

4. *Comparison of pens receiving wet and dry mash.*

Both pen 6 (wet mash) and pen 3 (dry mash) received yeast and both were fed under normal conditions. It appears that direction of difference is in favor of the wet mash in most cases although the differences are not biometrically significant.

Practically the same results appeared in pens 4 and 7. Pen 7 received wet mash and yeast which was fed at 15 per cent level. Pen 4 received the same ration with the exception that the mash was fed in the dry state.

The mash fed (wet) to pen 8 seemed to be somewhat superior to the same mash fed (dry) to pen 5, inasmuch as the eggs were somewhat larger in size and weight and the body weights of birds were somewhat greater. We believe this to be due to the heterogeneity of these birds. Egg production, however, was practically the same in both pens.

5. *The experiment with hens.*

The yeast fed pen ate more food and laid eggs that were somewhat longer and heavier than the control pen. It is also apparent that the individuals in the yeast fed group maintained their body weights better.

It seemed advisable to examine the data with reference to financial returns. Examination of Table II would seem to indicate that there was little or no financial advantage in feeding the yeast product. When these data were treated biometrically, it was found that many of these differences were not significant.

TABLE II.—INCOME OVER FEED COSTS FOR TEN MONTHS

Pen No.	Mash	Per Cent Yeast	Total Feed Cost	Vale of Eggs	Income Over Feed Cost
			PULLETS		
1	dry	25	$1.327	$4.308	$2.981
2	dry	none	0.993	3.899	2.886
3	dry	25	1.283	3.248	1.965
4	dry	15	1.162	3.752	2.590
5	dry	none	1.009	3.292	2.283
6	wet	25	1.288	3.584	2.296
7	wet	15	1.201	3.707	2.506
8	wet	none	1.019	3.424	2.405
			HENS		
9	dry	25	1.415	3.880	2.465
10	dry	none	1.116	3.717	2.601

The prices of the various ingredients entering into the rations were taken from the actual cost of these ingredients delivered at the experiment station. Throughout the year the feed prices varied. Hence, it was necessary to compute the cost of feed for each pen by the month. The price of the following articles did not change throughout the year; for example, yeast remained at $0.0625 per pound, beef scrap at $0.0475 per pound, grit at $0.95 per 100 pounds and oyster shell at $1.05 per 100 pounds. All eggs were sold in New York City. Therefore, the prices upon which these calculations are based are the average New York net prices per month throughout the year.

In analyzing the differences of the various groupings of pens with regard to income over feed costs, the following points are evident:

Pen 1 showed a slight advantage in income over feed costs over Pen 2, but did not give a corresponding increase in profit by feeding high levels of yeast product. That is, there is no significant difference in the income over feed costs.

Pen 2 showed an increase in income over feed costs (by the use of electric lights) as compared with Pen 5 (without electric lights) but the difference was not of sufficient magnitude to be significant.

Pen 1 (with electric lights) showed a significant increase in income over feed costs as compared with Pen 3 (without electric lights) but Pen 3 suffered from disease more severely.

Pen 4 (with 15 per cent yeast product) showed a significant difference in income over feed costs as compared with Pen 3 which received 25 per cent yeast product in the ration.

Pen 3 did not show a significant difference in income over feed cost as compared with Pen 5 which received no yeast product. Here the odds were in favor of Pen 5 although they were not significant.

Pen 4 (with 15 per cent yeast product) showed a difference in income over feed costs in its favor as compared with Pen 5. These results approach significance.

Pen 7 (receiving 15 per cent yeast product) did not show a significant difference in income over feeds costs as compared with Pen 8, which received no yeast product. Here the odds are in favor of Pen 7, but are not significant.

Pen 6 (receiving 25 per cent yeast product) failed to show a significant difference in income over feed costs as compared with Pen 8. Here the odds are in favor of Pen 8. but are not significant.

There was no significant difference in income over feed costs between Pens 6 and 7. indicatitng that there was no advantage in feeding the larger amount of yeast product.

There was no advantage in feeding wet mash as compared with dry mash as far as income over feed costs was concerned. Two cases were in favor of wet mash (Pens 6 and 8) and one case (Pen 4) was in favor of dry mash. No significance can be attached to the results.

There is no significant difference in income over feed costs in either of the pens in which hens were maintained.

SUMMARY

1. Eight pens of White Leghorn pullets, containing forty-five pullets per pen, were fed wet and dry mash rations with and without a commercial yeast containing corn meal and under normal and artitficial lighting conditions. One pen of forty-five hens was fed the control ration while another pen of hens was fed the control ration supplemented with yeast.

2. The birds were arranged in as homogeneous and comparable groups as could be done under the conditions of the experiment.

3. Data were collected on (1) food intake (2) body weight (3) egg production (4) total weight of eggs (5) length and width of eggs (6) weight of individual eggs and (7) income over feed costs.

4. The data were treated biometrically using Student's and Pearson's formulas.

5. The experiment was conducted for ten months.

6. An epidemic of roup and canker ran through the entire colony during the experiment.

CONCLUSIONS

1. Pullets and hens, not forced for egg production and under natural conditions, employing a good ration with adequate yards, did not appear to need an additional amount of vitamin B to maintain their egg production and body weight.

2. In most of the experimental groups, the pullets and hens which were fed yeast ate slightly more food and produced more eggs. The hens and pullets which received yeast maintained their body weights in a more satisfactory manner than those which did not receive the yeast.

3. Yeast had a distinctly beneficial effect when White Leghorn pullets were forced to undergo a longer feeding and exercising period by the use of artificial light. Under these conditions pullets developed better, laid more eggs and the eggs were larger and heavier. It is possible under these conditions that the vitamin B content of good poultry rations is not adequate for maximum results.

4. While cost data were collected throughout the entire experiment, we have purposely omitted any discussion of such data for the reason that it is evident that the yeast was fed at levels much higher than would be necessary in practical poultry practice.

The Pennsylvania State College

School of Agriculture and Experiment Station

Department of Agricultural Extension

M. S. McDOWEL, DIRECTOR

October, 1924 Leaflet No. 21

The Preparation of Cider Vinegar

By H. B. Pierce

The most important part of making cider vinegar lies in the use of clean utensils and barrels. Old barrels, kegs, or other containers, as well as the cider press itself, harbor many types of undesirable bacteria and molds unless all articles of equipment have been properly cleansed and sterilized before use. These undesirable bacteria and molds produce the wrong kind of fermentation and instead of changing the apple sugars into alcohol and acetic acid, they may be changed into worthless products, thereby producing an unmarketable vinegar, low in acid and poor in flavor.

Before using, the cider press should be scrubbed thoroughly with soap and hot water, and finally rinsed with plenty of boiling water. The barrels should be rinsed out and scalded with boiling water or live steam, if the latter can be obtained.

Avoid the use of rotten and dirty apples because (1) undesirable bacteria and molds will be introduced and bring about the improper changes mentioned in the preceding paragraph, and (2) the sugar content of the apples decreases when they begin to decay, which will limit the amount of alcohol formed. The amount of sugar varies from 7 per cent to 15 per cent according to the variety of apples, degree of ripeness, etc. Green apples contain scarcely any sugar, but much starch, whereas ripe, sound apples contain the maximum amount of sugar. If the apples have been frozen, use them before they start to spoil.

The juice of the apples, as it comes from the cider press, should be filtered through clean muslin or several thicknesses of cheese cloth, into clean barrels or containers. If barrels are used, do not fill them over two-thirds to three-fourths full. Cover the bung holes of the barrels with cheese cloth or muslin, tacked down to keep out flies and dirt. A cotton plug may be inserted in place of the muslin or cheese cloth. If jars or crocks are used, keep them well covered to prevent any contamination with vinegar flies by tying a piece of muslin over the top of the container. Alcohol cannot form unless the apple juice has a sufficient amount of air available; for this reason barrels should be placed on their sides, as this leaves a greater surface exposed to the air. Additional holes, one at each end of the barrel, may be bored to allow greater circulation of air. These, too, should be covered with cloth or plugged with cotton.

To hasten the formation of alcohol, one cake of yeast may be added for every five gallons of cider. This should be done in the following manner: Boil about one pint of water to destroy undesirable bacteria and molds and allow the water to cool. Dissolve the required number of yeast cakes and pour the mixture into the cider. Tip the barrel several times to distribute the yeast uniformly through the cider, and during the fermentation process keep the cider in a warm place at a temperature of from 65° to 70° Fahrenheit. This greatly shortens the fermentation period compared to that required in the average cellar, and yet the temperature is not high enough to cause undesirable fermentations or the loss of alcohol by evaporation.

Some people believe that cider can be left in the original container during the whole fermentation process, while others believe the cider should be removed in from four to six weeks and the sediment and sludge washed from the barrels. While the former process may be satisfactory, it often happens that this sediment may undergo decomposition and spoil the flavor of the vinegar.

Two to four quarts of old vinegar, containing more or less "mother," may now be added to the hard cider in each barrel. Do not add old vinegar to the fresh apple juice as alcoholic fermentation must take place first. The barrels or containers should be kept in a warm place, the same as during alcoholic fermentation. The barrels should be left undisturbed for three to five months. At the end of that period the vinegar may be drawn off and placed in clean barrels or containers. These containers should be filled and well stoppered to prevent any contamination. Under these conditions the vinegar should keep perfectly.

No cider vinegar may be sold in Pennsylvania if it contains added drugs or acids. Water may not be added to the vinegar if it reduces the acetic acid to less than four per cent by weight. The only safe rule for the farmer to follow, if the vinegar is to be sold, is to make his vinegar from the apple juice and add no water. Each cask or barrel must bear a statement similar to the following, "Cider Vinegar made by (Name) .... (Address) ...." This information may be placed on the cask or barrel by the use of stencils, or by tacking a card bearing the information on each container. If these specifications are observed, no chemical analyses of the vinegar need be made.

When vinegar has been frozen, no portion of it should be drawn off for sale until the frozen portion has been thawed and the entire contents of the vat or barrel thoroughly mixed so as to restore the homogenous character of the material. When vinegar freezes, there is a separation of the acetic acid and water, the acetic acid freezing first and settling to the bottom of the barrel, with the result that the upper portion of the liquid would not contain the legal amount of acetic acid, while the lower portion would contain acetic acid in excess of the legal requirement.

To summarize: Some of the conditions which produce low grade vinegars are:

1. **Poor apple juice.** This means the juice does not contain enough sugar to produce the required acetic acid. The use of unripe apples, partially decayed fruit, the normal juice which has been watered, or pomace which has been soaked with water and pressed a second time, gives a poor juice.

2. **Storing the apple juice at too low a temperature.** Quite often the cider is placed in a cool cellar, where the process of vinegar formation may require two or three years. Ignorance of this fact may result in the maker placing his product on the market before it is sour (acid) enough.

3. **Lack of air.** Acetic fermentation requires air. If barrels are filled too full, or if the bung is tightly stoppered, air is excluded and acid is not formed.

4. **Unsanitary conditions.** These favor the growth of undesirable bacteria and molds which destroy the vinegar. After the vinegar is sour enough, the containers should be completely filled and tightly corked.

5. **Freezing.** This causes a separation of the acetic acid and water. The water portion may be drawn off, leaving a large amount of acetic acid in the barrel. The liquid drawn off, therefore, would have a low acid content.

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THE TEACHING OF BIOLOGICAL CHEMISTRY*

R. Adams Dutcher, Department of Agricultural and Biological Chemistry,
Pennsylvania State College, State College, Pa.

A few weeks ago Dr. Gordon, your Chairman, wrote me, asking if I would be willing to lead a discussion of the topic which is now before you, *i. e.*, "The Teaching of Biological Chemistry." Without giving the matter further thought, I foolishly consented, thinking that I would discuss the teaching of Biological Chemistry from the view point of the so-called "Agricultural Chemist." Had I known that Biological Chemistry was to be discussed in contradistinction to Agricultural Chemistry, I am sure that I would have demurred, for the reason that I have had little or no experience outside of the Agricultural field. When I discovered my error it was too late to notify your Chairman, consequently I decided that I would do my best to raise a few questions which might stimulate discussion. I must, however, discuss the teaching of Biological Chemistry from the standpoint of the biochemist who is employed in a School of Agriculture, and who is confronted with problems quite different from those which confront the biochemist in a medical college.

If you will pardon me, I am going to take my own institution, The Pennsylvania State College, as an example and show you some of our problems. Our organization is such that all biological sciences, zoölogy, botany, plant physiology, plant pathology, bacteriology, etc., are in the School of Agriculture. Furthermore all Biological Chemistry is taught in the Department of Agricultural and Biological Chemistry. We have therefore, five general types of students for which our courses are designed, *i. e.*, students majoring in (1) Agriculture, (2) Home Economics, (3) Pre-Medicine, (4) Agricultural and Biological Chemistry and (5) Sciences other than chemistry. I am enumerating these facts because I feel that opinions are often influenced by environmental conditions and in our case our opinions and methods are bound to be influenced by the types of students we teach and their respective needs.

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* Read in the Division of Chemical Education of the American Chemical Society at the Ithaca Meeting, September 11 (1924) during the discussion of the report by the Committee on "The Teaching of Agricultural Chemistry." See This Journal, **1**, 177 (1924).

Before continuing with my discussion it is only fair to state that I have been unable to differentiate between Agricultural and Biological Chemistry. The longer I work in this field the more I am coming to the belief that Agricultural Chemistry courses are undergoing a gradual transition. In fact it is safe to say that there are very few courses offered in Agricultural Chemistry today that can be classified as strictly agricultural. Aside from certain courses emphasizing the purely analytical phases, practically all agricultural chemistry is biological chemistry.

It was not so many years ago that a course in Agricultural Chemistry meant the routine estimation of crude protein in feed, solids in milk, or phosphorus in a fertilizer. These and similar determinations were ends in themselves. Today they are but tools and their old importance is gradually diminishing. I do not wish to leave the impression that I am belittling analytical training for I consider it one of the most valuable types of training the student can obtain. I wish to emphasize, however, that we are paying greater attention to the biological applications of chemistry. Our modern student in Agriculture, Home Economics and Medicine is coming to us with excellent foundations in botany, zoölogy, physiology, bacteriology and genetics. As a result the biochemist has found it his opportunity as well as his duty to stress the applications of his science.

Furthermore our progressive departments in animal husbandry, agronomy, horticulture and dairy husbandry are demanding specialized courses in chemistry with requests that we give their students all the theory that they can assimilate but that we keep their respective lines of specialization in mind with regard to illustrative material. As a result we (and others) have very successful courses in phytochemistry, soil chemistry, dairy chemistry, enzyme chemistry, and the chemistry of foods and nutrition. All of these, without exception, are taught from the viewpoint of the biochemist but with biological and agricultural applications. One of our very successful new courses "The Physical Chemistry of the Cell" is an example of the trend of the times. A "pure" physical chemist could not teach a course of this type, for his students would possess a knowledge of biology superior to that of their teacher. The same principle holds true in many other lines of biochemistry. In such courses, an instructor must not only be well trained in chemistry but he must be better versed than his students in the vocational phases or the biological phases of the subject.

The same general viewpoint is maintained in our courses for girls in home economics except that food values, food preparation, health and hygiene are the pegs upon which we hang our fundamental chemistry and biology.

It is no longer possible for a physiological chemist trained in animal biochemistry to teach a course in phytochemistry to juniors or seniors for the students may have had excellent training in botany, mycology, plant physiology and plant pathology. Biochemistry is becoming specialized.

Our viewpoints are changing very rapidly regarding the teaching of dairy chemistry. Now we recognize milk as a biological fluid. We are interested in its biological origin. We not only analyze it but we separate and study its proteins, we examine it with the ultramicroscope, the cryoscope and the viscosimeter. We are interested in iso-electric points and enzymes. This is not the dairy chemistry of a decade ago and I am wondering if we can call it "Agricultural Chemistry."

In physiological chemistry the same vocational or specialization interest is catered to. There is a decided tendency in all of these courses to emphasize more theoretical or pure chemistry than was the case ten or twenty years ago. The chemistry and physics of the colloidal state is influencing classroom and laboratory work.

In most of our biochemical courses we find that a judicious mixture of qualitative and quantitative laboratory work gives the best results. Students lose interest, as a rule, in purely qualitative work. Quantitative work gives the best training so far as precision, neatness and accuracy are concerned but well planned qualitative tests, isolations and syntheses offer excellent opportunities for introducing many new principles and theoretical applications in a short period of time.

Classroom material is presented as often as possible from the standpoint of research and, above the sophomore year, with the frequent use of original literature. In my experience every child is born with the inherent curiosity which prompts the demolition of the watch to see its wheels go round and although most of this attitude of mind is lost by the time the student reaches college, most of our students become very much interested in finding out how scientific investigations develop. This is one type of outside work that usually brings results.

In conclusion I will say that it will never be possible to standardize teaching nor is it advisable to try. One instructor will obtain excellent results by the use of methods and materials that would fail in the hands of another. We can all agree, within certain limits, concerning the subject matter to be covered in various courses, we can exchange ideas and experiences and benefit thereby, but that is as far as we will be able to go.

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With the Compliments of the Author.

TOXICITY OF THE CASTOR BEAN *

F. L. FOLLWEILER, M.S.
AND
D. E. HALEY, Ph.D.
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Certain cases of sensitiveness to the poisonous action of the castor bean have been reported by Arnold,[1] Bernton,[2] Robbins[3] and Snell.[4] A number of such cases developed in connection with experimental and research work involving the use of the castor bean and its products in the Department of Agricultural and Biological Chemistry of the Pennsylvania State College. Much work has been done in this department with the castor bean, particularly with the ricinus lipase.

REPORT OF CASES

Case 1.—Mr. S., an instructor in the department, during the fall of 1922 contracted what appeared to be a severe cold, marked by violent and continuous sneezing and coughing, with irritation and edema of the mucous membranes of the nose, throat, eyes and ears, together with a dull headache and watering of the eyes. The condition seemed to grow worse instead of improving, and he gradually developed congestion of the bronchi, with painful and oppressive breathing, accompanied by a wheezing sensation and expectoration of mucus. Swelling and stiffening of various joints, particularly those of the arms and legs, developed, accompanied by pains similar to those experienced in grip, nocturnal paroxysms of suffocation accompanied by labored breathing, cold perspiration, vomiting, violent aching of the jaws and teeth, which necessitated the removal of several of the latter, marked intestinal pains, and alternate diarrhea and constipation, together with some fever. Throughout this entire period there was a gradual loss in weight marked by extreme prostration.

Mr. S. noted that his condition was improved on entering the open air, and during the period from Saturday noon until Monday morning, when he remained away from the laboratory. On entering the laboratory, however, there seemed to be an increase in the severity of his symptoms. One day, while he was conducting a class in laboratory work, a student began sifting some castor bean lipase preparation, and Mr. S. immediately became so affected that it was almost impossible for him to breathe. He at once suspected that he was sensitive to the castor bean. Later, during a period when the castor bean problem was not being worked on and the laboratory was thoroughly cleaned, a rapid improvement in his health ensued, and after several months the symptoms entirely disappeared.

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* Contribution No. 13 from the Department of Agricultural and Biological Chemistry of the Pennsylvania State College.

[1] Arnold: Science **59:** 577 (June 28) 1924.

[2] Bernton, H.: Am. J. M. Sc. **165:** 196 (Feb.) 1923.

[3] Robbins, W. J.: Science **58:** 305 (Oct. 19) 1923.

[4] Snell, W. H.: Science **59:** 300 (March 28) 1924.

CASE 2.—Dr. A., in company with several students, spent an afternoon cleaning up the small laboratory in which the castor bean lipase preparation had been sifted. Considerable dust was raised during the process of cleaning, and after the task was completed several of the students engaged in the operation experienced marked irritation of the mucous membranes of the nose, throat and eyes, this condition being accompanied by violent sneezing, coughing and burning sensations. The following morning Dr. A. was taken violently ill, complaining of intense abdominal pains or cramps and cold sweats, extreme vomiting, diarrhea and burning of the stomach, headache accompanied by severe prostration, bone pains with considerable stiffness of the joints, sneezing and coughing with irritation of the mucous membranes, and more or less fever. These symptoms extended over a period of several days, after which they gradually and completely disappeared.

CASE 3.—Mr. F., a medical student, became interested in the study of the castor bean lipase. He spent several days preparing the ricinus lipase from the castor beans, by hulling and macerating them, extracting the fat by the use of ether, and finally drying, pulverizing and sifting the finished product. After a time he experienced a dull headache, later accompanied by occasional sneezing and coughing, with a peculiar dry burning sensation in the nose and throat, and a slight itching of the skin at points at which the castor bean material had been brought in contact with his hands and arms. These symptoms gradually became more severe, the coughing and sneezing becoming extremely violent and continuous, while the irritation and edema of the mucous membranes of the nose, throat, ears and eyes increased very rapidly. His condition gradually became worse. A congested condition of the bronchi developed, accompanied by a hard, suffocating, continuous cough, hoarseness, raw feeling in the throat, pains in the chest, and nocturnal paroxysms of suffocation occurring usually about 2 a. m., with difficult breathing and cold perspiration. The symptoms were accompanied by severe attacks of abdominal pains, alternate diarrhea and constipation, violent inflammation and aching of the teeth and jaws, bone pains, stiffness and soreness of the joints, severe headache and extreme prostration. It was noticed that there was improvement in his condition when he was in the open air and when away from the laboratory, this improvement being

followed by an intense aggravation of the symptoms when he again returned to work in the laboratory.

Finally the symptoms became so violent that the attending physician deemed it necessary to order immediate withdrawal from the laboratory and advised abandoning the research problem. Within a short time after this had been done, a marked improvement was noted in the patient's condition, followed by a gradual disappearance of his symptoms in less than a week, with the exception of the congested condition of the bronchi, which required from five to six weeks for complete disappearance.

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VITAMIN STUDIES.

XI. INORGANIC BLOOD PHOSPHORUS AND BONE ASH IN RATS FED ON NORMAL, RACHITIC, AND IRRADIATED RACHITIC DIETS.*

BY R. ADAMS DUTCHER, MATTIE CREIGHTON, AND HENRY A. ROTHROCK.

(From the Department of Agricultural and Biological Chemistry, Pennsylvania State College, State College.)

(Received for publication, September 10, 1925.)

In the course of our studies on the effect of commercial methods of evaporation on vitamins A, B, and C in cow's milk, it appeared desirable to take advantage of our supply of experimental milks and extend our studies to include the antirachitic factor. Inasmuch as x-ray and histological facilities were not available, it became necessary to resort to chemical methods, as a means of ascertaining the physiological conditions of our rats, so far as calcium and phosphorus deposition is concerned.

In the preliminary feeding trials we used the Sherman-Pappenheimer ration (1) but this was finally discarded in favor of a "yellow corn ration" devised by Dr. Harry Steenbock of the Wisconsin Experiment Station. We feel that the latter ration has given more satisfactory results because of its superior protein complex and its higher content of vitamins A and B. This ration will be described at another point in this paper.

Examination of the literature showed that little work had been done on the chemical composition of blood and bones of rats at different ages and, inasmuch as the rachitic rations, used by other investigators, have varied in their chemical composition, particularly with reference to calcium and phosphorus, it was deemed advisable to make a preliminary study on our rats under the conditions existing in our laboratory with the view of determining the composition of blood and bone at various ages, using the new Steenbock rachitic ration.

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* Published as contribution No. 18 of the Department of Agricultural and Biological Chemistry.

It was decided that determinations of inorganic blood phosphorus should be made because previous investigations (2) have shown that a knowledge of the fluctuations of inorganic phosphorus in blood might be helpful in making our interpretations.

Work of McCollum (3), Steenbock (4), and others has indicated that the determination of the ash content of bones is even more helpful in arriving at conclusions regarding the utilization of calcium and phosphorus by the rat. For this reason, it was decided that this determination should be made, using the extraction method of Steenbock.

EXPERIMENTAL.

While it has not been our general practice, it became necessary to resort to group feeding for the reason that it was difficult to obtain sufficient blood from a single rat, especially during the early stages of the experiment when the rats were small. Consequently, each cage contained not less than four rats, while some cages, which contained rats which were to be removed from the experiment during the first few weeks, contained a larger number, the total number of animals being 103. The rats were placed on experiment at an age of about 21 days, no animal weighing less than 35 or more than 40 gm.

Three types of rations were fed; Ration A being the normal ration which is used for our breeding stock; Ration C being Steenbock's yellow corn ration, and Ration B, Steenbock's ration which had been irradiated for 15 minutes with the Cooper-Hewitt "Uviarc" quartz mercury vapor lamp at a distance of 2 feet.

Ration A consisted of the following:

	per cent
Wheat	45.0
Corn	17.0
Rolled oats	4.9
Linseed meal	8.0
Bran	2.5
Dried meat scraps	5.0
Powdered skim milk	10.0
Sodium chloride	1.0
Calcium carbonate	1.5
Ferric citrate	0.1
Alfalfa meal	5.0

These ingredients were ground and fed with fresh whole milk (*ad libitum*) and fresh greens were fed twice each week.

Ration C, Steenbock's yellow corn ration, consisted of the following ingredients:

	per cent
Yellow corn (ground)	76.0
Wheat gluten	20.0
Calcium carbonate	3.0
Sodium chloride	1.0

As stated above, Ration B was the same as Ration C except that it was irradiated with ultra-violet light.

The experiment was conducted for 8 weeks and at least four animals were removed each week from each group represented by the rations described. When the rats were removed at the end of each week, they were anesthetized and the blood was obtained by cutting the jugular vein. The individual blood samples of each experimental group were added together and set aside in a refrigerator overnight for the determination of inorganic phosphorus. The femurs were removed, scraped free from adhering tissue, and composited into single experimental samples, air-dried, and extracted with alcohol and ether. After extraction, the composite weekly samples of bones were dried in an oven at 100°C. until free from moisture, and ashed. All ash percentages were calculated on the basis of extracted dry bone. The colorimetric method of Benedict and Theis (5) was used and the inorganic phosphorus was calculated to mg. of phosphorus per 100 cc. of serum.

DISCUSSION.

Charts 1, 2, and 3 are self-explanatory. It will be noted in Chart 1 that the normal rats, on Ration A, grew unusually well, as might be expected on a ration of that type. It has been our experience that our rats are somewhat larger at 3 weeks of age than rats reared in some laboratories. This may be due to the unusually varied diet that we feed to our breeding colony.

While the rats receiving Ration B did not grow well, we were unable to observe any symptoms of rickets or other abnormalities. These rats appeared normal and remained active until the end of the experiment. The rats receiving Ration C, the rachitic ration, began to show rachitic symptoms in about 2 weeks and none of them lived beyond the 7th week.

The inorganic blood phosphorus in the normal group, Ration A (Chart 2), tended to remain fairly constant at a level of about 10 mg. per 100 cc. of serum. The rachitic bloods showed an immediate fall in inorganic phosphorus, which continued for about 3 weeks, when it rose slightly, due to the fact that the animals had gradually lost appetite, resulting in a lowered food intake. This agrees with the observations of McCollum and coworkers (6) and Cavins (7) who found that fasting tended to cause an increase of inorganic blood phosphorus when the level of this blood constituent is relatively low. It is of interest to note that ultra-violet irradiation had the effect of causing the inorganic phosphorus to lie midway between the normal and the rachitic levels.

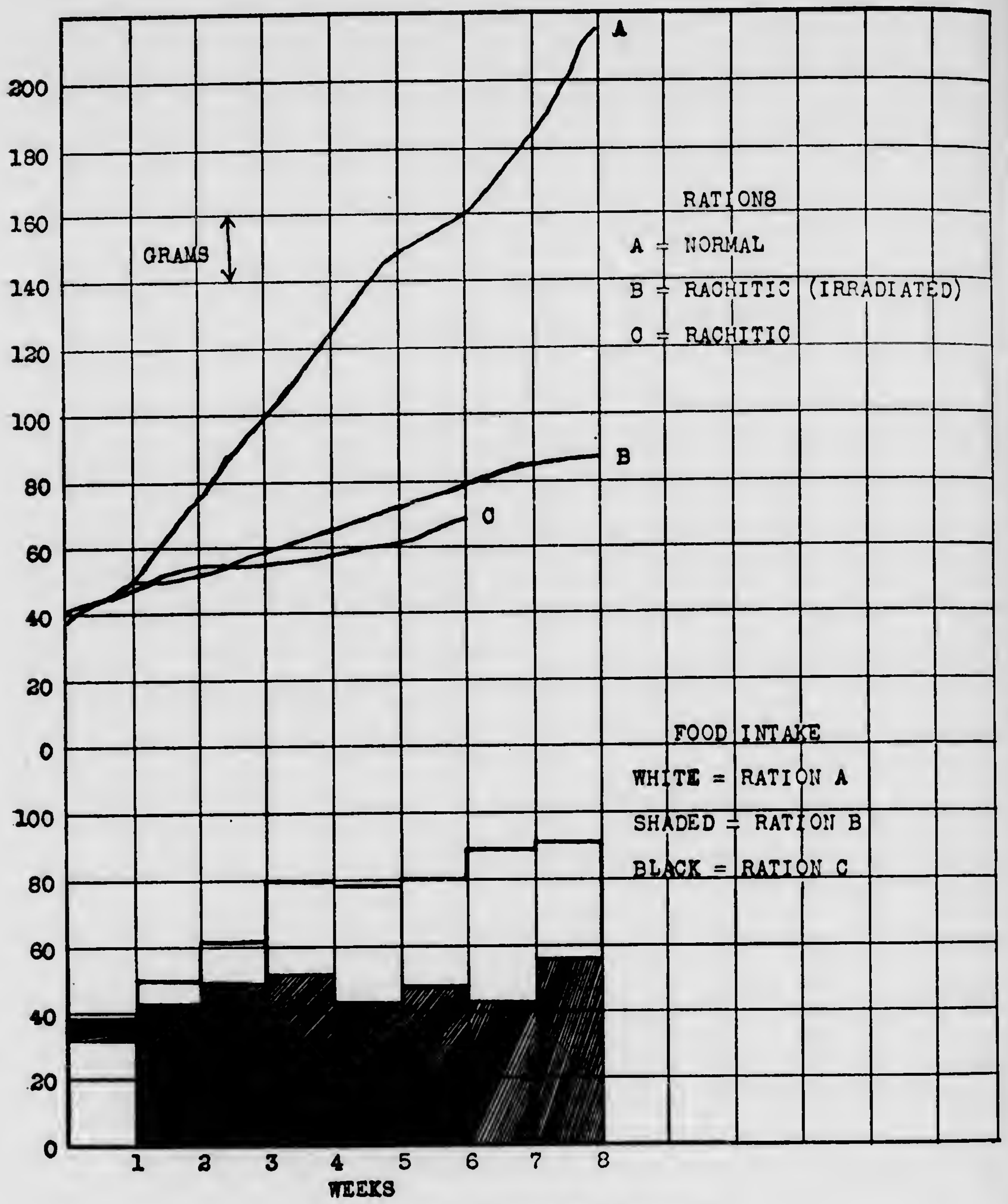

Chart 1. Curves of growth.

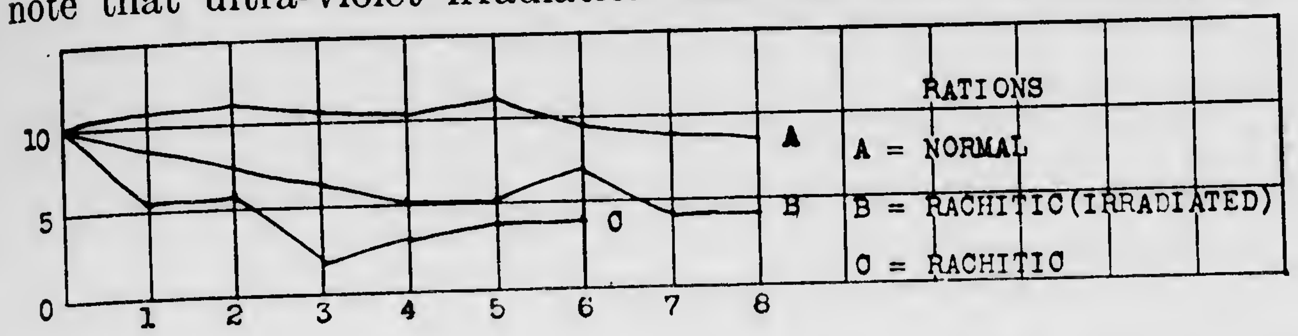

Chart 2. Inorganic blood phosphorus expressed in mg. per 100 cc. of serum.

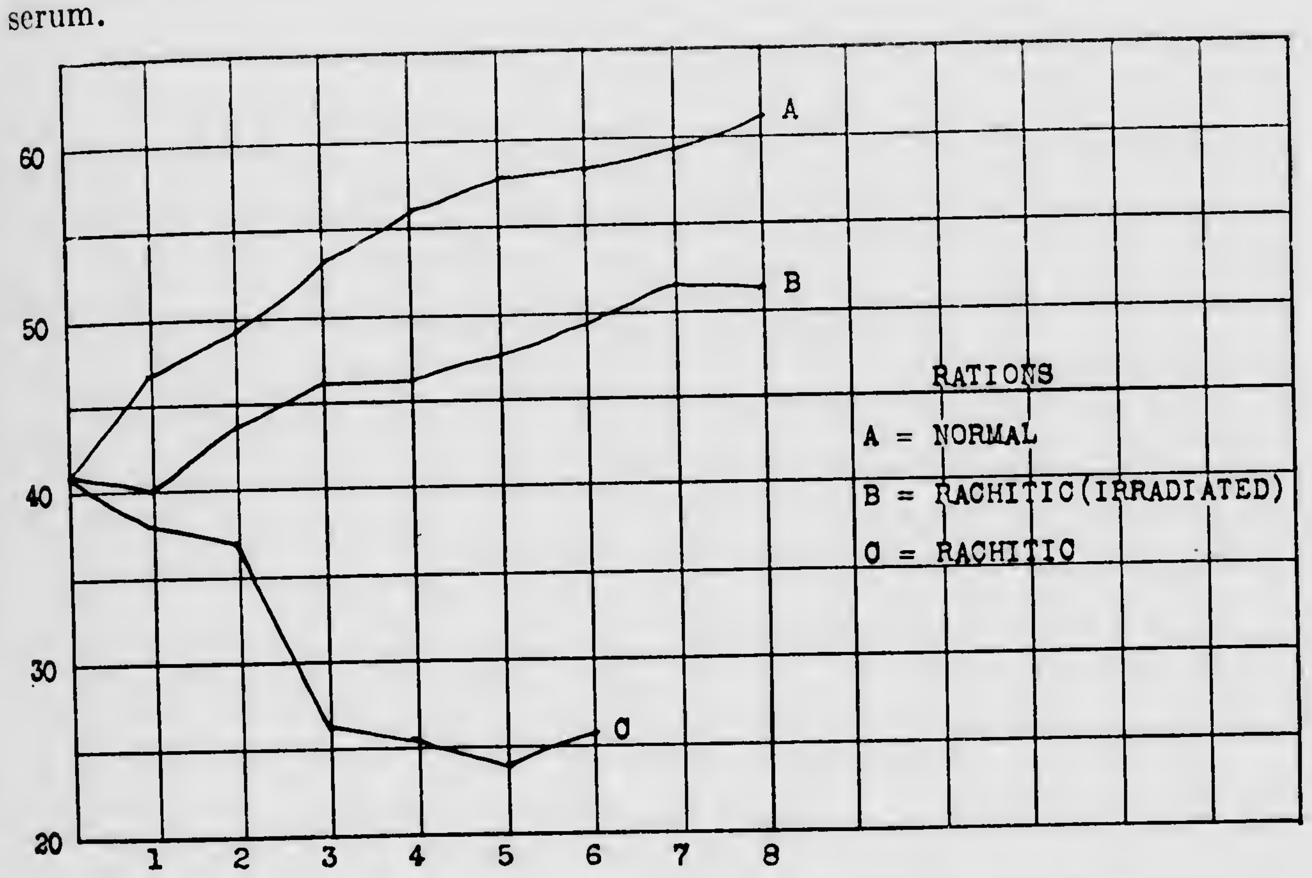

Chart 3. Bone ash expressed in per cent of dry, extracted femurs.

Examination of Chart 3 shows a similar situation with regard to the bone ash in the femurs. It is of importance to note that the percentage of ash rises steadily in the normal animals as age progresses. Our figures compare very favorably with those of Steenbock (4) in this regard. When the rachitic ration is irradiated there is no abrupt fall in bone ash; on the contrary, the tendency seems to be for the ash to rise in a rather consistent manner. When the ration is not irradiated, however, the percentage of bone ash, in extracted bone, drops immediately and continues to do so until fasting affects the situation, which in our work did not occur until after the 3rd week.

Another point which merits emphasis is the fact that calcium and phosphorus deposition (Ration C) seems to be inhibited from the beginning of the feeding experiment. This leads us to raise the question as to the ability of the rat to store the antirachitic factor. If storage of this factor does occur we would not expect the blood phosphorus and the bone ash to fall, for a time, until the storage supply was depleted. This does not seem to be the case in our rats, however, and we are led to believe that the rat is not capable of storing significant amounts of the antirachitic factor.

It would appear, therefore, that these chemical methods should be very useful in making accurate estimations, in a relatively short time, of the antirachitic potency of our common foods. It offers a quantitative method of unusual interest, for we should be able to find, by feeding experiments, the minimum amount of any food which will prevent an appreciable fall of inorganic blood phosphorus or bone ash below the normal level of a normal 40 gm. rat or the minimum amount of any food which will bring about normal deposition of mineral matter in bones.

CONCLUSIONS.

In normal rats, weighing between 35 and 40 gm., the inorganic blood phosphorus was 10 mg. per 100 cc. of serum and during an 8 week feeding period, the tendency was to maintain this level, falling slowly to 8 mg. per 100 cc. of serum at the end of 8 weeks. The percentage of ash in the dry extracted femurs of the same normal rats was 40 at the beginning of the experiment and increased steadily, during the 8 week period, to 62 per cent.

When rats received Steenbock's rachitic ration, inorganic blood phosphorus dropped from 10 mg. to 1.6 mg. in a period of 3 weeks. After that period it rose slightly, due to fasting. The ash of dry extracted femurs of the rachitic rats fell from 40 per cent to 26.5 per cent in 3 weeks and finally fell as low as 24 per cent.

When Steenbock's rachitic ration was irradiated with ultraviolet light the inorganic blood phosphorus and the percentage of bone ash fell midway between the normal and rachitic groups.

It would appear that the rat does not possess the ability to store the antirachitic factor in significant amounts for the reason that calcium and phosphorus deposition is hampered from the very beginning of the experimental feeding period.

The method as outlined offers an opportunity to make a quantitative study of the antirachitic properties of food materials, using as a standard the least amount of any food which will prevent fall in the percentage of bone ash, below that possessed by the young normal animals at the beginning of the experiment or the minimum quantity of food required to bring about optimal deposition of mineral matter. Work is now in progress with the view of attempting to define the conditions under which this method may be applied in the study of the antirachitic properties of food materials.

The writers wish to take this opportunity to acknowledge the generosity of Dr. Steenbock in allowing them to use his rachitic ration, prior to its publication by him.[1]

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VITAMIN STUDIES

XIII. VITAMIN B IN EVAPORATED MILKS MADE BY VACUUM AND AERATION PROCESSES*

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AND
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The investigational work described in this paper is the first of a series of coöperative studies being made at the Agricultural Experiment Station of the Pennsylvania State College on evaporated milks. This work is a continuation of a larger project concerning the various factors influencing the vitamin content of milk and dairy products. Former work has dealt with dietary factors (1) (2), heat and pasteurization (3), oxidation (3) (4), and commercial drying by the spray process (5).

In the past practically all of the data available, relative to the vitamin content of evaporated milks, have been obtained on samples the past history of which has not been known. Furthermore, few attempts have been made to compare the evaporated milks with the raw milks from which they were made. As a result few authoritative data have been obtained concerning the influence of commercial evaporation processes on the stability of the various vitamins.

The present studies were undertaken with the view of preparing evaporated milks under conditions approximating, as closely as possible, the conditions used in commercial practice and comparing these products with the original milk from which they were made, to eliminate the effect of any fluctuation in vitamin content that may take place in raw milk obtained from various sources and at different seasons of the year.

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* Technical Paper No. 407 of the Agricultural Experiment Station. Published as Contribution No. 22, Department of Agricultural and Biological Chemistry, and Contribution No. 12, Department of Dairy Husbandry. Received for publication February 25, 1926.

THE EVAPORATED MILKS

The raw milk, from which the evaporated milks were made, was obtained, daily, from the college herd and was fed to the experimental animals within a few hours after the morning milking, although a portion of the raw milk represented the milking of the previous evening.

Two types of evaporated milk were prepared, the first being manufactured by the vacuum process in a Rogers 18-inch copper vacuum pan and the other by the aeration method, passing a current of air through the hot milk. The object in using the methods just described was to determine, if possible, the susceptibility of vitamins to oxidation during the evaporation process. We also desired to study the effect of subsequent sterilization, for sterilization plays an important rôle in the manufacture of commercial evaporated milks. At this point it is well to emphasize the fact that the writers do not claim to have duplicated commercial methods of manufacture but the attempt has been made to approximate commercial conditions as closely as possible.

EVAPORATED MILK MADE BY THE VACUUM PROCESS

While the raw milk was fed daily to the experimental animals, it was impossible to manufacture daily "batches" of evaporated milks. As a result the evaporated milks used in this study were manufactured every three weeks.

Prior to the evaporation process, milks from the evening and morning milking were mixed thoroughly and samples were taken for analysis. These samples were analyzed for titratable acidity, total solids and fat. The specific gravity was recorded also.

In the case of milks made by the vacuum process, the raw milk was pre-heated in the hot-well until the temperature reached 200° to 210°F., after which it was drawn into the vacuum pan. The temperature in the pan ranged from 125° to 135°F., averaging about 130°F. The average vacuum obtained was about 23 inches. Unfortunately the available supply of raw milk was occasionally limited, with the result that the charges in the pan were not constant in volume; as a consequence, the time of evaporation varied from forty-five minutes to one hour and fifteen minutes.

When the milk in the pan had reached a concentration of 7° to 9° Beaumé at 120°F., it was homogenized at 1800 to 2000 pounds pressure. Specific gravity, solids and fat were determined on the homogenized evaporated product after which one half of the evaporated milk was placed in sterilized bottles, of 250 cc. capacity, and these bottles were placed on ice until needed. Each bottle usually contained sufficient milk for one day's feeding.

The other half of the evaporated milk was also placed in similar bottles, plugged with cotton and sterilized at 240°F. for ten minutes and at 225°F. for five minutes, using a laboratory autoclave. These bottles were also placed on ice until needed for feeding purposes.

By the use of the analytical data, described above, it was possible to dilute the evaporated milk samples with distilled water until the diluted milks contained solids and fat equal to that of the raw milk from which they were made. All evaporated milks were fed daily in the diluted state in direct comparison with fresh raw herd milk.

EVAPORATED MILK MADE BY THE AERATION METHOD

During the early part of the investigational work the aerated evaporated milks were made in a Ruff evaporator of the commercial type, consisting of a heated revolving drum which was partly immersed in the milk. Air was driven through the heated milk by means of blower pipes. The experimental milk, used in this study, was produced by the college dairy herd that furnished the local market with certified and grade A milk. As a result there were periods when the amount of milk available for evaporation purposes was so limited that the quantity of milk was insufficient to immerse the heated drum. For this reason the use of the Ruff evaporator was abandoned and air pipes were connected with a glass lined pasteurization tank of small capacity and the subsequent aerated evaporated milks were made by this method, heating the milk by means of steam.

The hot-well temperatures were maintained at 145°F. and the condensing temperatures ranged from 130° to 155°F. On account of the varying quantities of fresh milk, the time of condensing varied from forty-five minutes to one hour and fifteen minutes.

From this point the evaporated milks made by the aeration methods were treated the same as those made by the vacuum process; i.e., homogenized and a portion sterilized by the fractional sterilization method.

During the entire period the college herd was confined to the barn and yard, without pasture, and received a ration relatively constant in composition with the exception that some green hay was fed during two months in the fall. The ingredients of the ration, expressed in parts per 100, were as follows:

	Per cent
Wheat bran	12
Barley	12
Oats	8
Hominy	24
Cotton seed meal	12
Linseed meal	16
Peanut meal	16

Some minor modifications were made in the proportions of the above ingredients during the latter part of 1924. During the entire period mixed hay and silage were fed, as roughage, with exception of the two months mentioned above, when green hay was fed.

It will be seen, therefore, that the vitamin B intake of the herd was about as constant as can be expected under ordinary feeding conditions.

It will be noted that five types of experimental milks were fed, which we have abbreviated for convenience (on the figures) as (*a*) R, i.e., raw milk; (*b*) V.E., i.e., vacuum evaporated milk (not sterilized); (*c*) V.E.S., i.e., vacuum evaporated milk which had been sterilized; (*d*) A.E., i.e., aerated evaporated milk (not sterilized); and (*e*) A.E.S., i.e., aerated evaporated milk which had been sterilized.

THE FEEDING EXPERIMENTS

A total of 289 rats have been used in this work, although the charts do not represent that number, owing to the fact that it was necessary to repeat some of the early work on account of the observations of Steenbock, Sell and Nelson (6) and Dutcher and Francis (7) that vitamin B studies are inaccurate unless provisions are made to eliminate the possibility of the rats having access to their own excretory material. This error was eliminated in part at least, by changing our feeding technique and adopting the use of false cage bottoms consisting of wire screen (3 or 4 meshes to the inch). Since the publication of the above mentioned findings Salmon (8) and Smith, Cowgill and Croll (9) have published experimental data substantiating the work of Steenbock, Dutcher and co-workers and showing that this change in feeding technique is necessary if the best results are to be obtained. In our publication (7) we stated that we were of the opinion that "the rat is limited in its ability to store vitamin B." As a result it was decided that little could be gained by using the "curative" method of feeding and, consequently, all rats described in this paper were fed the various experimental milks from the beginning of the experimental period, rather than adding the milk to the diet after vitamin deficiency had become evident, which is the practice in the "curative" method.

RAT RATION AND FEEDING TECHNIQUE

The ration consisted, in parts per 100, of casein 18, salts 3, agar 2 and dextrin 77. Four drops of a potent cod liver oil were fed daily, separate from the ration, to insure an adequate intake of vitamins A and D. Iodine was furnished in the drinking water. The casein was prepared by precipitating skim milk according to the Zoller method (10). The washings, with acidified water (pH 4.8), never numbered less than 12 and between washings the casein was pressed as free of water as possible, in a cheese press.

The water washings were followed by 5 or 6 alcohol washings, allowing each "batch" to remain in contact with alcohol for at least three hours. This treatment served to extract any traces of vitamin B remaining in the casein and, incidentally, removed sufficient water to prevent spoilage of the casein on standing. After treatment with alcohol the casein was spread in thin layers on glass shelves in an air drier and air (at room temperature) was driven over the casein to remove the alcohol.

The casein was then treated with ethyl ether until 50 cc. of the ether extract left no fatty residue, upon evaporation. The casein was then air dried, heated in an air oven at 120°C. for twelve hours and ground to a powder. This product has been used in all of our feeding work and has been found to be free from vitamins A, B and C.

The dextrin was prepared by autoclaving corn starch in the moist state, in the presence of 0.4 per cent citric acid. After drying in a steam closet, until the product was thoroughly brittle, it was ground to a fine powder.

The salt mixture was McCollum's salt mixture No. 185 and the agar was a high grade product of the type used in bacteriological work.

As is the custom in this laboratory, all experimental animals were confined in individual cages and food consumption records were obtained on every individual. These food intake records were used as a guide in the interpretation of data but are not of sufficient importance to be included in the charts.

Each experimental group contained not less than 8 individuals and some groups (particularly the groups receiving 12 cc. of milk) contained as many as 16 rats. Males and females were distributed equally in all groups to eliminate the influence of sex on the growth curves obtained in averaging the individual growth curves in each group. On account of the large number of animals, it was thought best to conserve space by presenting "group averages" rather than growth records of each individual. It was felt, also, that the data are more easily interpreted by consulting averages for each group.

All feeding experiments were conducted for one hundred forty days. In order that seasonal variations, if any, might be eliminated all animals were not placed on experiment at one time but groups were added from time to time during the experiment.

When this was done, however, an equal number of rats were placed in each experimental group.

Osborne and Mendel (11) have shown that a minimum of 16 cc. of milk, produced under conditions existing at New Haven, were needed to furnish sufficient vitamin B for the normal growth of rats. The work of Kennedy and Dutcher (2), working with milk produced under feeding conditions existing in St. Paul, found that they could obtain satisfactory growth of rats on as little as 10 cc.

With these results in mind, it was planned to feed the milk, described in this paper, at levels below and above 10 cc. Consequently, the milk was fed at 6, 8, 10 and 12 cc. levels with the hope that we would find a "threshold level" at which point vitamin destruction (if any) by heat or oxidation would be most marked.

DISCUSSION

All of the experimental data have been averaged and are summarized in figures 1 and 2. None of the animals receiving 6 cc. of milk grew normally, although growth was quite satisfactory.

No outstanding differences seem to exist between the groups receiving 6 cc. of raw milk and those receiving equivalent amounts of vacuum evaporated (V.E.) milk. Furthermore, the sterilization of this evaporated milk did not seem to affect its nutritive properties. The rats receiving 6 cc. of the aerated (A.E.) evaporated milk did not grow so well but the differences are not marked. Sterilization of this evaporated milk did not bring about differences sufficiently large to merit emphasis.

What has been said for the rats receiving 6 cc. of milk can be said, also, for those fed at the 8 cc. level. In fact, no effect of the additional 2 cc. of milk can be noted on any of the curves.

When the volume of milk is increased to 10 cc. it is possible to note a distinct improvement in all groups. It is difficult, however, to note any effect of milk treatment in any of the groups receiving more than 6 cc. of milk.

When the volume of milk, fed per rat, was increased to 12 cc. fairly normal growth was obtained, showing that the Pennsylvania herd milk was slightly superior, in vitamin B content, to that described by Osborne and Mendel (11) but it seems slightly inferior to the Minnesota milk described by Kennedy and Dutcher (2).

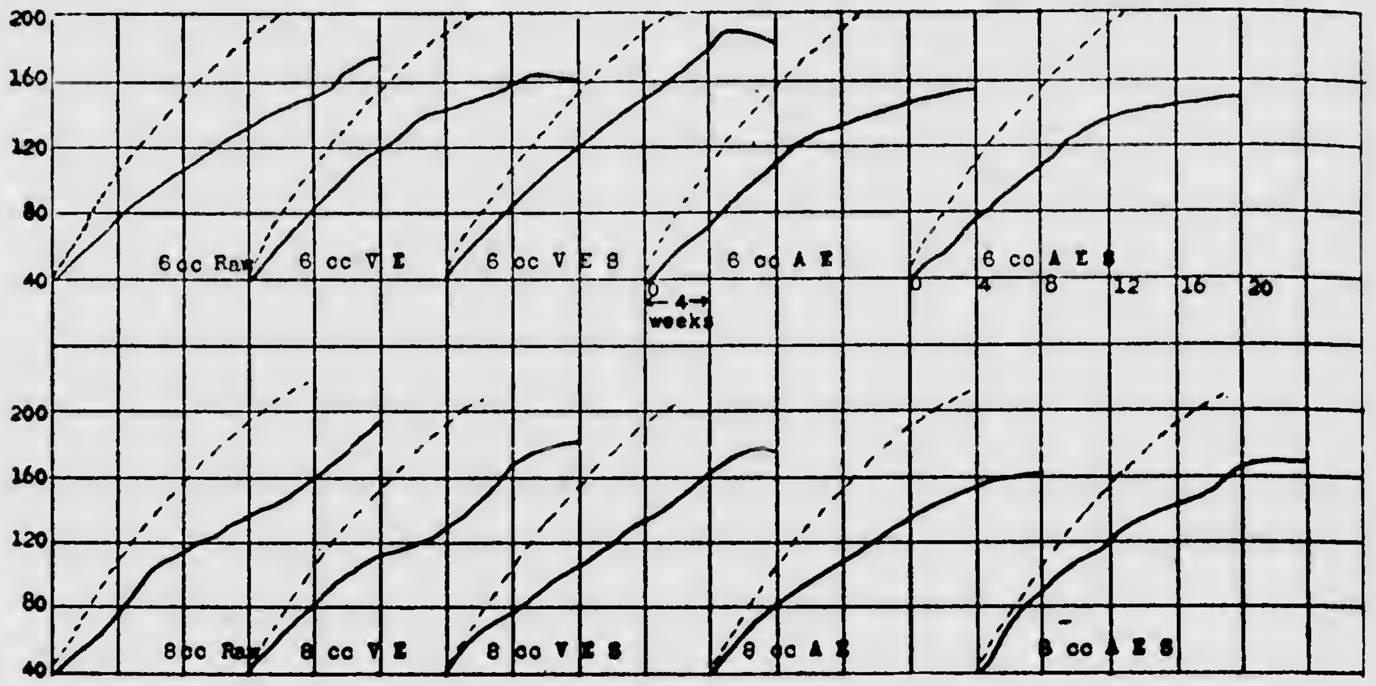

Fig. 1

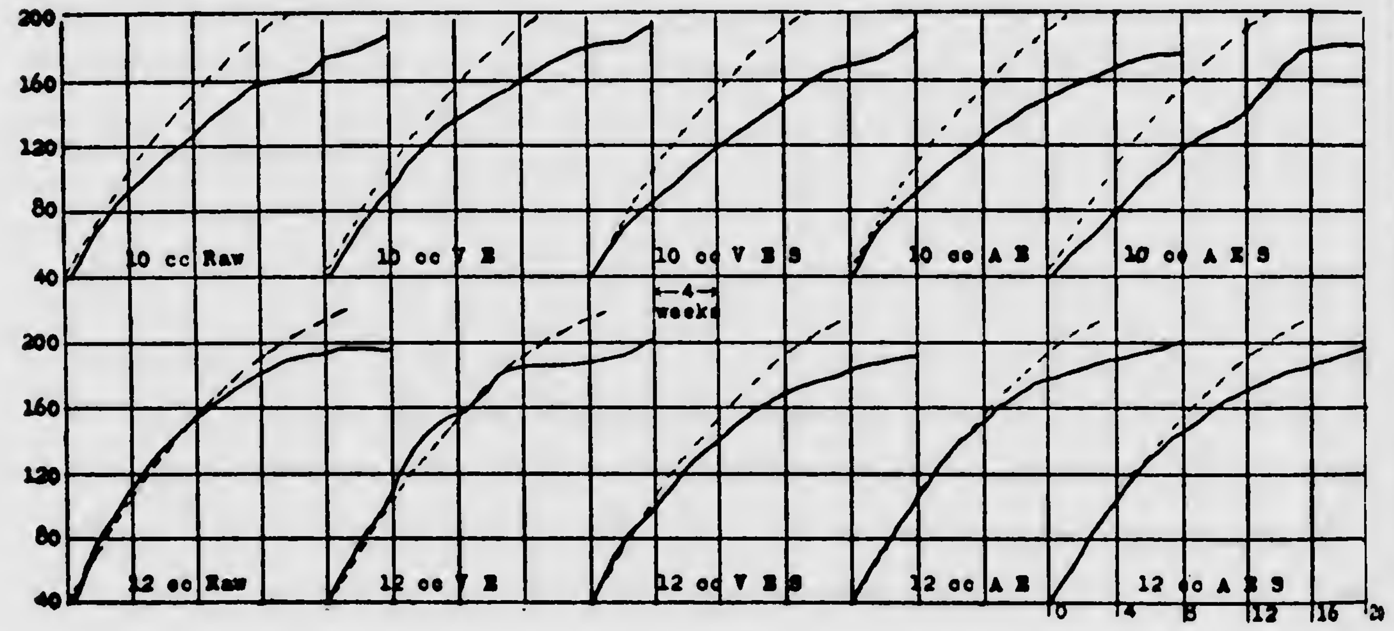

Fig. 2

SUMMARY AND CONCLUSIONS

Evaporated milks have been made by vacuum and aeration methods, approximating commercial manufacturing conditions as closely as possible. These milks were sterilized and compared, by animal experiments, month by month, with unsterilized evaporated milk as well as with the raw milk from which they were made. This was done to study the effect of heat and oxidation on the stability of vitamin B. The evaporated milks were diluted with distilled water until they possessed the same composition as the raw milk and all experimental milks were fed at 6, 8, 10 and 12 cc. levels. No evidence of vitamin B destruction could be noted except in the groups fed at the 6 cc. level. In these groups there was some evidence that aeration or oxidation and heat may have brought about slight destruction of vitamin B. The destructive effect, however, was so slight that it is doubtful if it can be considered to be of nutritional importance.

We are forced to conclude, therefore, that vitamin B is not readily destroyed by the evaporation methods described and only under unusual conditions would we expect the vitamin B deficiency of commercial evaporated milks to be due to methods of manufacture.

Experimental work is now in progress to attempt to determine the stability of vitamin A during the processes of evaporation and sterilization by the methods described.

The writers wish to acknowledge the assistance of Miss Mattie Creighton, who assisted materially in the experimental phases of the work, and the hearty coöperation of the herdsman, Mr. P. D. Jones.

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VITAMIN STUDIES.

XIV. THE INFLUENCE OF ULTRA-VIOLET LIGHT ON THE ANTI-RACHITIC PROPERTIES OF PURIFIED RATIONS USED IN THE STUDY OF VITAMIN A.*

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(Received for publication, June 5, 1926.)

For a number of years it has been the practice in most laboratories to determine the vitamin A content of food materials by feeding a purified vitamin-free basal ration to growing rats and supplementing this ration with wheat embryo or yeast preparations and weighed or measured quantities of the food to be investigated. The basal ration used in this laboratory consists, in parts per 100, of casein 18, agar 2, salt mixture 3, and dextrin 77. Yeast pellets, weighing 0.5 gm. (made by pressing Fleischmann's special dried yeast in a pill press) are fed daily to insure an adequate supply of vitamin B and iodine is furnished in the drinking water.

In all but our very recent work the casein was prepared by precipating skim milk according to the Zoller method (1). The washings, with acidified water (pH 4.8), never numbered less than 12 and between washings the casein was pressed as free of water as possible in a cheese press.

The water washings were followed by five or six alcohol washings, allowing each batch to remain in contact with alcohol for from 3 to 12 hours. This treatment served to extract any traces of vitamin B remaining in the casein and, incidentally, removed sufficient water to prevent spoilage of the casein on standing.

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* Published by permission of the Director of the Agricultural Experiment Station as technical paper No. 410 and contribution No. 23 from the Department of Agricultural and Biological Chemistry.

After treatment with alcohol the casein was spread in thin layers on glass shelves in an air drier and air (at room temperature) was driven over the casein to remove the alcohol.

The casein was then treated with ethyl ether until 50 cc. of the ether extract left no fatty residue, upon evaporation. The casein was then air-dried, heated in an air oven at 120°C. for 12 hours, and ground to a powder. This product has been used in all of the feeding work and has been found to be free from vitamins A, B, and C.

The dextrin was prepared by autoclaving commercial cornstarch in the moist state, in the presence of 0.4 per cent citric acid. After drying, in a steam closet, until the product was thoroughly brittle, it was ground to a fine powder.

The salt mixture was McCollum's Salt Mixture 185 (2) and the agar was a high grade product of the type used in bacteriological work.

Recently we have attempted to dispense with the above tedious and expensive method of casein preparation. At present we are obtaining very satisfactory results by percolating a commercial dry casein with alcohol and ether, using a technique practically identical with that described above, the final product being heated, in shallow pans, in an electric oven at a temperature of 120°C. for 12 to 15 hours. The latter treatment is added as a precautionary measure to insure the destruction of the last traces of vitamin A.

Prior to the discovery (3) that some rachitic food materials could be rendered antirachitic by means of ultra-violet irradiation, no satisfactory means were available to insure an adequate supply of the antirachitic factor—in fact, it is doubtful if it had occurred to many workers that vitamin A investigations were being vitiated by the fact that rickets was complicating the problem.

In March, 1925, we placed three groups of rats on the basal ration just described. The first group received the basal ration; the second group received the basal ration and the rats were irradiated for 10 minutes (daily) with the Cooper Hewitt Uviarc lamp at a distance of 2 feet; the third group received the same treatment except that the ration was irradiated for 30 minutes and the rats received no light treatment.

While xerophthalmia developed in all groups, it was clear that irradiation had delayed the fall in body weight that we had formerly observed. Furthermore, we concluded that irradiation of the ration possessed many advantages over direct irradiation of the animals, particularly with reference to convenience and economy of time.

To test the matter further, we started new experimental groups and fed raw milk at various levels, using *both types* of technique, *viz.* with and without irradiation. It was discovered that less milk was required to produce the same increase in growth, if the ration was irradiated with ultra-violet light. This work will be described in a later paper.

In the meantime we had collected data on bone ash and inorganic blood phosphorus in rats at different ages, which led us to suggest (4) that it was possible to determine the calcifying properties of food materials, using bone ash as an index of calcifying potency.

The present paper endeavors to apply this technique to the study of our purified basal ration with the view of definitely answering the question as to which ingredient is responsible for the increased well being of our experimental animals and which ingredient has the greatest antirachitic or calcifying potency after irradiation.

EXPERIMENTAL

Using the same technique as described in our former paper (4), 66 rats were placed on the casein-dextrin basal ration at 21 days of age. Inasmuch as previous experience had shown that the maximum rachitic effect becomes manifest, in our rats, at about the 3rd week, it was decided that we would determine the ash in the femur bones at the 14th and 21st day of the experiment. In the present investigation no attempt was made to collect data on the fluctuations of inorganic phosphorus in the blood.

All rats (see Table II) received the same basal ration with the exception that Group 7 received a diet in which 2 parts of dextrin were replaced with 2 parts of corn oil.

The complete ration (Group 2) was irradiated after mixing. In all other rations, except the control ration (Group 1), the ingredients were irradiated separately, before mixing.

On the 14th day of the feeding period three rats were removed from each group, anesthetized, and the femur and humerus bones were cleaned, dried, extracted, and ashed. Due to an error, both types of bone were made into composite samples for each individual rat; consequently, the (femur) ash percentages in Table I represent standards suggested in our previous paper (4), while the data in Tables II and III represent mixtures of femur and humerus ash.

The data in Table I are self-explanatory. Using our former standards, which are summarized in Table I, and consulting the data in Table II, it seems evident that the irradiation of the complete purified basal ration (Group 2) has brought about a degree of calcification that can be considered subnormal but not what would be considered rachitic from a clinical standpoint. It is also apparent that the pruified basal ration (Group 1, without irradiation) produces a rachitic condition. When the ingredients were irradiated singly and added to the other ingredients, which had not been irradiated, it is evident that casein (Group 3), agar (Group 4), and the salt mixture (Group 6) played little, if any, part in the stimulation of the calcification process, while definite stimulation was brought about by the action of the ultra-violet light on dextrin (Group 5) and corn oil (Group 6).

TABLE I.

Standards Originally Established (Femur Only).

Condition of animals.	Average bone ash 14th day.	Average bone ash 21st day.
	per cent	*per cent*
Normal	49.00	53.00
Subnormal, no evidence of rickets	43.50	46.55
Severe rickets	37.00	26.60

In order that we might assure ourselves that this method was capable of yielding consistent results the experiment was repeated, using 24 rats fed the same ration except that dextrin was the only ingredient which received ultra-violet irradiation.

In this experiment four rats were taken from the experiment each week for 5 weeks, Group 1 being removed at the beginning of the experimental feeding period, Group 2 at the end of the 1st week, Group 3 at the end of the 2nd week, etc.

The results of this experiment are summarized in Table III.

These results compare surprisingly well with the data in Table II, considering the number of variables not under control. It will be noted that irradiated dextrin, in the respective trials (Tables II and III), produced 46.87 and 46.10 per cent of bone ash on the 14th day and 48.03 and 49.00 per cent on the 21st day. In other words, additional proof was obtained that dextrin, made from commercial corn-starch and fed in the proportions described, has the property of assuming antirachitic properties when treated with ultra-violet light.

TABLE II.

Effect of Irradiating Various Ingredients in the Casein Dextrin Type of Ration.

Group.	Treated with ultra-violet light.	Total no. of rats.	Average bone ash (in three rats). 14th day.	Average bone ash (in three rats). 21st day.
			per cent	*per cent*
1	None	6	37.90	40.23
2	Complete ration	6	45.67	47.33
3	Casein	6	42.53	41.60
4	Agar	6	41.07	41.57
5	Dextrin	6	46.87	48.03
6	Salt mixture	6	40.63	40.77
7	Corn oil	6	46.83	50.80

TABLE III.

Effect of Irradiating Dextrin Only.

Group.	Days on experiment.	Average bone ash.
		per cent
1	0	40.80
2	7	43.75
3	14	46.10
4	21	49.00
5	28	54.50
6	35	54.20

Furthermore, it seems safe to say that this bone ash method, when standardized on controlled breeding stock of known dietary history, gives quite uniform results and should be useful in making quantitative studies of antirachitic food materials. For example, it would appear that, inasmuch as 2 parts of irradiated corn oil brought about the same degree of calcification as 77 parts of irradiated dextrin, it is possible to say that irradiated corn oil is about thirty-eight times as potent, as a calcifying agent, as is irradiated dextrin.

SUMMARY.

Additional data are submitted to show the usefulness of the bone ash method in determining the antirachitic potency of foods.

This method has been used to study the casein-agar dextrin type of diet used for the estimation of vitamin A, from which the following conclusions may be drawn. (*a*) That most, if not all, of the investigational work, in the past, has been vitiated due to the presence of rickets, in addition to the results of vitamin A deficiency. (*b*) That casein, agar, and salts in the proportions described have little, if any, effect on calcification, after irradiation. (*c*) That dextrin, fed at a 77 per cent level, made from commercial corn-starch possesses marked calcifying properties after treatment with ultra-violet light. (*d*) That irradiated corn or olive oil can be added, to advantage, to vitamin-free rations of this type when estimations of vitamin A are contemplated. (*e*) That irradiated corn oil is at least thirty-eight times more potent than irradiated dextrin, made from commercial corn-starch by the method described. (*f*) Our results are in general agreement with those of Steenbock and coworkers (5), who observed that antirachitic activation could not be induced in purified protein while starch became activated when treated with ultra-violet light.

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THE
Nitrogenous Metabolism of Pyrus Malus

*Some Results Obtained in Investigations on the Distribution of Nitrogen in Apple Trees During a Year's Cycle**

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INTRODUCTION

THE purpose of the present paper is to call attention to some of the results, obtained during the past four years, of investigations on the distribution of the various nitrogen groups in the leaves, the one-year, two-year and older branch growths, and the short non-bearing spurs of apple trees throughout a vegetative cycle, and to the manner in which these fractions are modified by the application of sodium nitrate.

These investigations had their origin in an effort to seek a physiological basis for a choice between spring or fall fertilization with sodium nitrate to apple trees. The solution of the problem was first attempted, giving due consideration to the experiences of the earlier investigators (2), by ascertaining if the course of absorption of the NO_3 radical could be determined throughout an entire vegetative period by means of qualitative tests on sections and the dialyzed saps of the fine roots, buds, and the one, two and three year old bark of mature and seedling apple trees treated with heavy applications of sodium nitrate. In the aerial parts, positive tests were given at one period and in one tissue only—in the leaf buds just as they were opening. These experiences were later confirmed by numerous quantitative experiments and are supported by Dr. Eckerson's preliminary work (4) on the reducing power of extracts from various parts of apple trees.

The reduction of nitrates in apple trees must occur, for the most part, in the fine roots. Accordingly, the course of absorption and the fate of the nitrogen applied as sodium nitrate must be sought in some of the numerous transformation products which the NO_3 radical undergoes. With the object of determining the nature and course of these transformations, extensive investigations were carried out *in vivo*, following methods consistent with modern standards of accuracy. Details of this work with the methods adopted will form the third paper of this series in Plant Physiology.

AMINO ACIDS AND AMINES IN RELATION TO GROWTH

That amino acids may play an important rôle in the metabolic processes of plants has been recognized since Emmerling's classical investigations in the eighties, and has been especially emphasized more recently by the investigations of MacDougal (7) and of Spoehr and McGee (11), who conclude that amino acids acting as catalysts (3) in accelerating with rate of carbohydrate katabolis, —either by forming salts with carbohydrates or by participating in the buffer processes—are the principal nitrogen fractions influencing the rate of growth. The former worked chiefly on synthetic bio-colloids *in vitro* and more complete shading was effected in the experiments of the latter than would occur for growth. Whatever course the synthesis and degradation of proteins follows it is certain that amino acids and amines are not the only substances taking part in the transformations; for ammonia, the amides, the polypeptides, the basic nitrogen compounds, and also certain unclassified compounds—the so-called "rest" nitrogen—also play a rôle.

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The writer's investigations *in vivo* show that much further work is necessary before the true function of amino acids and amides is known, and that the "rest" or unclassified nitrogen compounds may also have an important function. Caution must, therefore, be exercised not to emphasize the part played by amines and amides to the neglect of these "other" nitrogen compounds which do not respond to the tests in any scheme thus far developed for partitioning the complex nitrogenous compounds of plants.

The Problem of Sampling

The work of Harley (5) and of Tufts (13) and others, has emphasized the fact that the problem of sampling of fruit trees for chemical analysis is not a simple one. There is need for much further information with respect to the selection and proper sampling of the parts of trees to be examined. Such work is rendered necessary not only because the different parts of a fruit tree appear to behave as independent units (1, 6) but also because of the variations in composition from the tip to the basal part of the shoot. For these reasons it is essential to apply the fertilizer uniformly around the root system, to take as uniform samples as possible of shoots or spurs, and to compare only trees of the same variety and age growing on a homogeneous soil. These conditions have been met in the experiments to be described. All samples were collected under the same conditions in the early part of the morning after a clear day; the results, therefore, should be strictly comparable.

Analyses of Fresh versus Dried Tissues

It has been urged by Roberts (9) that drying the tissues produces marked changes in the forms of nitrogen. However, under the conditions adopted in the present investigations, proteolytic enzyme activity was maintained at a minimum. This was shown by a comparison of analytical data on fresh and dried tissues from the same sample lot (12). Probably many of the changes reported in the literature as being due to proteolysis during drying at intermediate temperatures actually occur during the subsequent treatment with water, owing to failure to prevent reactivation of the enzymes. In the present experiments this was effectually accomplished by phenol.

Diurnal Changes

In the present investigation it was sought to avoid errors arising from diurnal changes by taking the samples at the same time of the day under similar atmospheric conditions. Experiments undertaken to ascertain the extent of diurnal fluctuations indicated that total water-soluble nitrogen was lower at the end of the day, but that amino acids varied and sometimes were higher and sometimes lower. These diurnal changes, however, were relatively small when compared with the seasonal fluctuations.

The 1923-24 Experiments

For the experiments of the first series a Stayman Winesap tree 15 years old, and in the "off" year, growing in sod in the experimental orchard of the Department of Horticulture, was used. It received an application of 5 pounds of $NaNO_3$ on April 7. Samples were taken on 11 dates between June 18, 1923, and June 11, 1924. For the purpose of the analytical work they were divided into (a) one-year branch growth, (b) two-year branch growth, (c) older (3-5 year) growth, (d) non-bearing spurs, and (e) leaves.

The Fractions Determined

The analyses were made on the water extracts of the dried tissues, which were preserved from bacterial action by toluene and, as already stated, from proteolytic enzyme action by phenol. The following fractions were determined: (1) total water-soluble N, (2) water-soluble non-protein N, (3) ammonia N, (4) free amino N, (5) amide N, (6) combined amino N, (7) humin N, and (8) "rest" N. Total nitrogen determinations were, of course, made on all samples.

Seasonal Distribution of the Nitrogen Fractions in the Branch Growth

As the buds are swelling the total nitrogen content of the one- and two-year branch growth increases, whereas, the nitrogen content of the older branch growth decreases. One might deduce from this that during this period the reserve proteins in the older growths are broken down and transferred to the more actively growing points. These processes are accompanied by a rapid rise in the total water-soluble and non-protein nitrogen and also in the free amino and "rest" N fractions; but the amide N decreases at this period. In the case of animals, the transference of reserve proteins to the tissues takes place chiefly as free amino acids; this may be the case also in the flowing sap of plants, although it may equally be argued both from the magnitude and direction of the "rest" N compounds that they, too, play a similar rôle.

The marked decrease in total nitrogen (Table I, p. 10) in the one- and two-year growths and older branch growth and the short non-bearing spurs, occurring between the beginning of vegetative growth and the period of blossoming, is accompanied by a rapid decrease in the total water-soluble and non-protein nitrogen and the free amino N, and a gradual increase in the amide N in the one- and two-year branch growths. The behavior of the non-bearing spurs is quite similar, although the variations in magnitude and, therefore, in the direction of the curves are greater.

During the period of active growth, the water-soluble nitrogen and the non-protein nitrogen, including the amino N, again increase, but the amide N decreases. This decrease in the amide N at this time would appear to be connected with the utilization of amide N in protein synthesis.

When active growth is over, both the total water-soluble and non-protein nitrogen, including the amino N and amide N, decline rapidly until fall translocation of nitrogen from the leaves begins, at which period the water-soluble nitrogen increases until the last sampling (Nov. 18), concomitant with which is a gradual increase in the amide N. Evidence has been obtained that this amide N is chiefly derived from asparagine.

THE DISTRIBUTION OF NITROGEN IN THE LEAVES

In the leaves, in general, the fluctuations of all the nitrogen fractions throughout the whole cycle vary inversely to their fluctuations in the branch growth. Since the results are expressed in percentages and not in absolute quantities of the weights of leaves and branch growth respectively, there may be some difficulty in interpreting these inter-relationships. It was found that the total water-soluble nitrogen, the amino N and the amide N paralleled the total nitrogen of the leaves throughout the cycle, but the "rest" N usually varied inversely as these other fractions.

One of the most interesting features of the curves of both leaves, branches and spurs is the close parallelism that exists between the total water-soluble nitrogen, the non-protein nitrogen, the free amino N and the combined amino N throughout the whole cycle.

THE 1924-25 EXPERIMENTS

In the second series of experiments the partition of nitrogen was examined in two Stayman Winesap trees of the same age and of similar growth behavior growing beside one another in the experimental orchard. One of these trees was fertilized with 10 pounds of $NaNO_3$ on April 21 and again with an additional 10 pounds on June 6.

It is easily possible to follow by means of the graphs for total water-soluble nitrogen, water-soluble non-protein nitrogen, mono-amino N and also amide N, the course of absorption of the $NaNO_3$. The various nitrogen fractions pursue such different courses in the two trees that, taken in conjunction with the results from the 1923-24 experiments, it is impossible to regard such differences as being due to any other cause than that arising from the application of $NaNO_3$ to the one tree and not to the other.

The course of the curves—especially for mono-amino, amide, and "rest" nitrogen—would indicate that the 10 pounds of nitrate on April 21 took nearly three weeks to reach the one-year branch growth, whereas the second application of 10 pounds on June 6 was effective within one week. The rainfall for the first period was 2.3 inches and the mean temperature 52.6°, and for the second period 0.32 inches and 61.1°, respectively. The absorption of the nitrate is characterized by a large increase in the total water-soluble nitrogen, the increase being chiefly in the amino N, amide N and basic N portion of the non-protein fraction of the fertilized as compared with the unfertilized tree in the leaves, the one-year and new wood growth. There appears to be no correlation between either temperature or sunshine or rainfall or humidity and any of the nitrogen fractions.

THE TOTAL NITROGEN

A composite curve showing the sum of the percentages of nitrogen found in the leaves and in the one to five year old branch growth throughout the cycle would suggest that nitrogen equilibrium may be just about maintained by the application of 5 pounds of $NaNO_3$ to a 15 year old Stayman Winesap tree growing in sod. Table I shows the results.

TABLE I—COMPOSITE TABLE SHOWING THE SUM OF PERCENTAGES OF THE TOTAL NITROGEN IN NEW GROWTH + ONE YEAR + TWO YEAR + OLDER WOOD + LEAVES

	Total Nitrogen (Including Leaves) Per Cent		Total Nitrogen (Wood Growth Only) Per Cent	
	Dry	Fresh	Dry	Fresh
June 18, 1923	3.866	1.705	2.210	0.986
July 16, 1923	4.332	1.767	2.463	1.078
Aug. 17, 1923	4.064	1.652	2.204	1.011
Sept. 17, 1923	3.851	1.686	2.105	0.971
Oct. 15, 1923	3.886	1.728	2.269	1.082
Nov. 20, 1923	4.149	1.827	2.579	1.242
April 4, 1924			1.925	0.911
April 21, 1924			2.508	1.158
April 28, 1924			2.302	1.032
May 13, 1924	4.987	1.563	1.475	0.686
May 22, 1924	4.364	1.227	1.766	0.821
June 11, 1924	3.805	1.612	1.805	0.941

These results are presented in the following graph (Fig. 1).

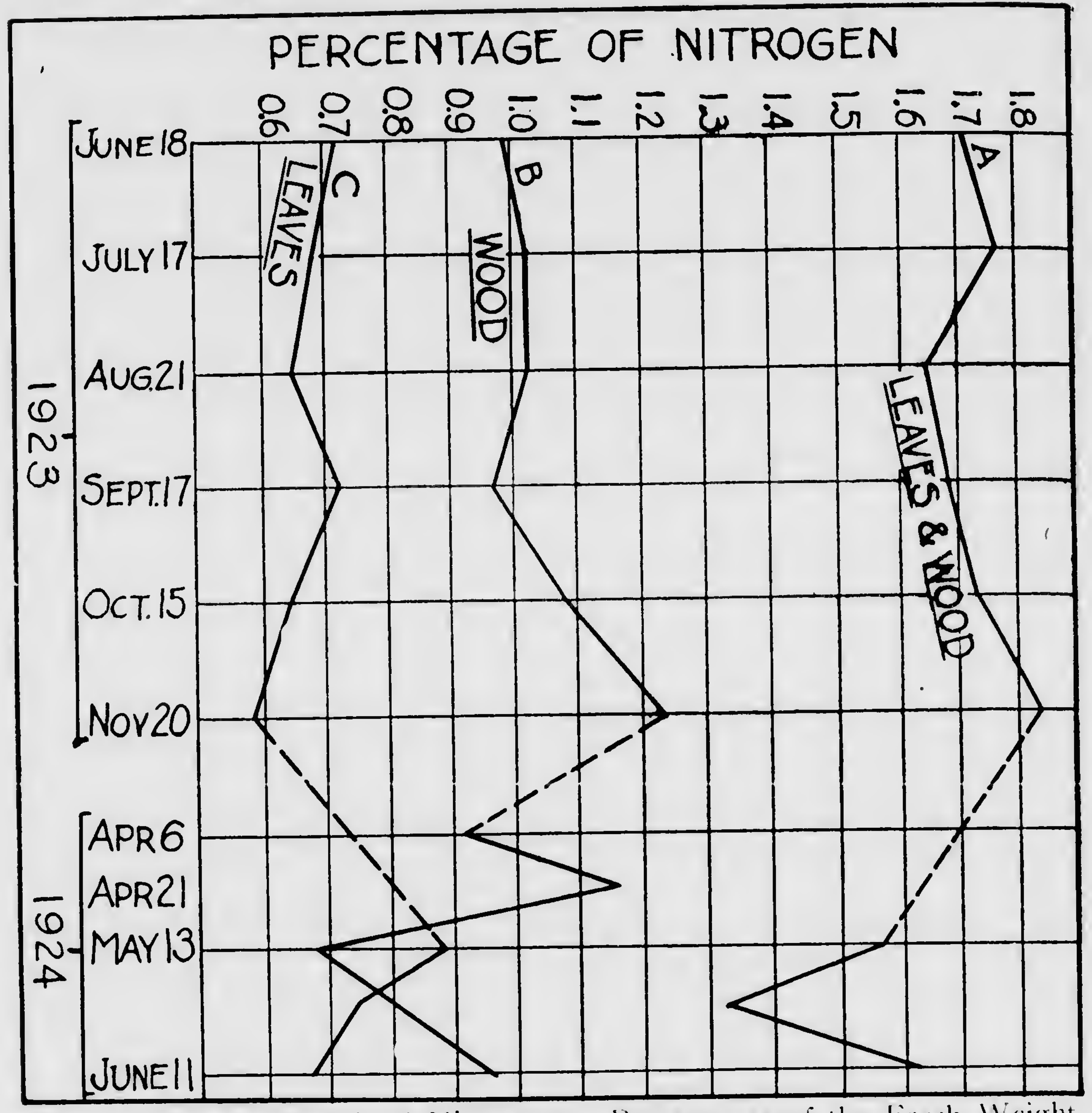

Fig. 1. Showing the Total Nitrogen as Percentages of the Fresh Weight of Material.

It is interesting to note the rate at which the nitrogen (which appears to be stored mainly in the one- and two-year branch growths and non-bearing spurs) is utilized in early Spring during the development of the buds. Thus, on the dry weight basis 2.51 per cent nitrogen is present in the total branch growth on April 21; but by May 13 only 1.47 per cent is present. This drop indicates that the development of buds and the formation of new leaves and fruit buds requires a very high expenditure of these stored nitrogen compounds. In some tissues over half the vailable supply is given up to the young shoots.

Although the young shoots made rapid growth from May 13 to June 11, the total nitrogen curve of the branch growth again rises rapidly, thus giving an index of the rate of absorption from the soil at this period. After this period the slope $\left(\frac{dy}{dt}\right)$ is practically zero, indicating probably a nitrogen equilibrium in the tissues concerned. Murneek (8), from the consideration of absolute quantities in spurs, arrives at this same conclusion.

The fall storage of nitrogen in the branches is clearly indicated in the curves. Nitrogen begins to be translocated from the leaves to the branches towards the end of September.

Discussion and Conclusions

These investigations have indicated that the seat of reduction of nitrates in Pyrus Malus is chiefly located in the fine roots and that the course of absorption and transformations of the nitrate radical can be followed by adopting specially refined methods in partitioning the various groups of the complex products formed by the very reactive nitrogen molecule. It must be admitted, however, that the solution of any of the problems with which practical horticulture is concerned by this means, involves, at the present time, laborious and time-consuming separations. To what extent the progress in our knowledge of protein chemistry will enable simpler methods to be applied can only be conjectured. Attempts by the writer to simplify the determination of amino nitrogen have thus far failed. Spoehr's simplified method for amino acids (10) has proved very erratic when applied to the water extracts of materials used in this investigation.

The close parallelism observed between the total water-soluble nitrogen and the free amino N throughout the cycle in all tissues, suggests interesting possibilities. Thus, if the amino acids are the chief fractions influencing growth and, consequently, the vigor of the tree, and if the law of mass action is applicable, the determination of the total water-soluble nitrogen may furnish a simple solution.

It must be borne in mind, however, that the unclassified or "rest" N compounds may play a rôle just as significant as any of the nitrogen partition products. Thus, during the fall storage of nitrogen the "rest" nitrogen compounds increases rapidly in the leaves and branch growth, clearly indicating its relation to protein degredation in the leaves and the probability of translocation of nitrogen in this form to the branches. The writer is planning to investigate the nature of these unclassified nitrogen compounds shortly.

In conclusion, it may be pointed out here that the analytical results on the trees in the apple-rim experiment described in the paper by Anthony and Thomas in these Proceedings have shown that the ratio of total carbohydrates to total N (or the ratio of starch to total N) and also the ratio of total N to total P_2O_5, are quite effective aids in diagnosis.

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Eliminating Some of the Variables in Apple Fertilizer Experiments

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In 1918 the high degree of variability secured in a large number of field tests of fertilizers in commercial orchards throughout the state led the Department of Pomology at State College to plan, in cooperation with the Department of Agricultural Chemistry, an experiment in which as many variables as possible should be eliminated and still permit growing the trees practically under field conditions.

Both the men originally responsible for this work, William Frear and J. P. Stewart, passed on to their reward before the plan was put into execution and the responsibility for the field and laboratory work fell upon the present writers. The horticulturist is responsible for the planting and field care of the trees, the biochemist for the methods of analysis and the analytical results.

As finally adopted, the problem was to study the influence of fertilizer and cultural treatments on the physiological functions of the apple. In other words, the attempt was to be made to eliminate or reduce to a minimum all the important variables except the fertilizer and to compare the fertilizer response under nonlegume sod and under annual tillage.

It was recognized at once that direct field planting would introduce too large a soil variable for this experiment so it was decided to sink boiler plate cylinders, or rims, 5 feet across and 5½ feet deep, into the ground and to place in these a uniform soil whose composition had not been influenced by the previous use of fertilizers. A Hagerstown silt loam from the same field used for the College experimental orchards was selected. It was removed by layers and placed in the rims in the same order after each layer had been thoroughly mixed. Before any soil was placed in the rims about 6 inches of coarse crushed limestone were placed in each rim and, as these were sunk in a limestone soil with very free downward drainage, it was thought this would inhibit capillary action.

As the soil was placed in the rims samples were set aside for analysis. These were studied for the usual soil elements and then given a careful minerological analysis. These results have been publishd by W. Thomas in Soil Science, Vol. XV, No. 1.

The soil was placed in the rims early in 1921 and was given one year to settle, during which time three green-manure crops of oats, buckwheat and rye were turned under. In spite of the uniformity of the soil at the time of placing in the rims, there were rather considerable differences in the growth of cover crops. This, we think, was due to the physical condition of the soil. The filling of the rims extended over a considerable time and there were variations in the moisture content during this time and consequently differences in the degree of compactness of the soil.

It was believed that one of the factors necessary for success with this experiment was to secure roots vegetatively propagated from a single parent. In 1920 this was not an easy condition to meet, but eventually, through the kindness of Professor R. G. Hatton of the East Malling Research Station in England, we were able to locate 150 individuals of Malling Type 12 which was considered nearly a standard in its vigor. Part of these stocks had just been separated from the mound layer and part had been one year in the nursery. These were planted in the spring of 1921 and two weeks later the trees from the nursery were whip-grafted to Stayman Winesap, the scions coming from a tree whose performance was known for two bud generations. The remaining stocks were budded in the summer from this same Stayman Winesap tree.

There were 42 rims placed in 7 rows of 6 each. In May, 1922, four rims in each row were planted with the whip-grafted trees, leaving the outer rims in each row unoccupied until the following spring, when the budded trees were ready. At first it seemed unfortunate to have trees of two ages in this experiment, but it has in the end proved a benefit. Certain results have been accentuated with increasing age of the trees and with two ages growing side by side it has been easier to evaluate this progressive influence.

In order to get the trees well established, all rims were treated alike until the summer of 1924. Each spring and until early summer they were hand cultivated and then a cover crop planted. The last of May, 1924, blue grass was seeded in half the rims of each row, the cultivation being continued in the other half until the last of July when the usual cover crop was planted in these. This differentiation had an appreciable effect upon the 1924 branch growth. In spite of this, at the end of that season this was one of the most uniform blocks of trees the writers have ever seen. A careful analysis by Student's method of the total branch elongation for 1924 showed only one row that might be considered significantly different from the adjoining rows.

The blue grass and timothy sod had been continued since 1924 on half the rims and the cultivation continued on the other half, but the cover crop practice has been changed slightly. Seeding the rims after the fertilizer treatments were started would have resulted in large differences in cover crop growth. Turning this under would have introduced a serious variable that was not wanted. An area approximately the same as the 21 cultivated rims and containing the same soil with which they are filled is seeded each fall with a rye cover. In the spring this is carefully dug and the dirt shaken from the roots. Tops and roots are divided into twenty-one equal parts and cut into the cultivated rims.

The first fertilizer applications were made in April, 1925. To avoid complications from impurities, chemically pure materials were used. The six trees of Row 1 received phosphorus in the form of calcium mono-phosphate at the rate of one pound of P_2O_5 per tree. Row 2 was to receive nitrogen as nitrate of soda at the rate of one-half pound of N per tree, but to avoid burning only two-thirds of the application was made at this time. Row 3 received the same phosphorus and nitrogen applications as 1 and 2 and in addition potassium as potassium sulfate at the rate of one-half pound of K_2O per tree. Row 4 is the untreated row. Row 5 received P and K; row 6 received N and K, and row 7 received N and P.

In a little more than a month the trees which received nitrogen could be picked out by the deeper color of their foliage. The surprising thing to us was that this held in the cultivated as well as in the sod area. This led us to believe that the use of the confining rim had brought about a root condition similar to that which would be found in a mature, closely planted orchard. The subsequent performance of the trees has served to strengthen this belief.

The nitrogen application, though only two-thirds of the full amount planned, caused such severe burning of the sod that it was not considered advisable to add any more that season. Even with this reduced application, by the end of the season there were pronounced differences between the trees which did and did not receive it, both under sod and under tillage. Among the trees which did not receive nitrogen, the sod trees could be picked out by their more yellow foliage. This was not true with the trees which did receive nitrogen.

The first application of fertilizers in 1926 was made on May 3 when one-third of all the materials was applied per tree. The second and third applications were made at intervals of a month. Even these reduced applications caused considerable sod burning, but the recovery was rapid and by the time the grass in the rims was ready to be clipped there were large differences in sod growth under the various treatments.

The tree growth differences first noted in 1925 were accentuated in 1926 as root crowding must have increased with the greater growth. The end trees in the rows which are one year younger did not show such great differentiation due to their lighter demand upon the soil.

Growing these trees in what has become a relatively limited area presents certain soil moisture problems that are difficult to solve. As long as the trees were under uniform treatment all were watered alike as the need arose. Now the responses to the different treatments have created a different moisture requirement. We are studying two methods of determining this requirement: one uses the evaporation from a Livingston porous soil point plunged into the soil; the other depends upon the weight changes of cedar pencils thrust into the soil. It will take another season to determine whether either method will enable us to maintain all rims at an optimum soil moisture.

The annual sampling and the subsequent analyses are discussed below. In addition to these it is expected that both the soil and the entire trees will be studied when the experiment is completed. The field records which are taken are trunk circumference, elongation of each branch, weights of all prunings, relative leaf color and average leaf weights of samples taken from the same situations on all trees, heights of grass growth and weight of clippings, number of blossoms and eventually number and weight of fruits and, of course, a record of all cultural operations. "Student's" method will be used for all statistical analyses.

One of the chief purposes for which the trees were grown under these controlled conditions was to supply material for analyses which might determine the various physiological responses to the conditions created by the experiment. Obviously with only three trees under a single treatment the size of the sample must be small. For several years two serious problems have been occupying most of the time of the chemist associated with this work: what types of analyses would present a picture of the physiology of the tree in sufficient detail to be capable of interpretation and how could these analyses be secured with the small amount of material available? Some of the results of these preliminary studies have already been presented by W. Thomas at this meeting and will be given in greater detail (including methods of analysis) in several future numbers of Volume 2 of "Plant Physiology."

At the end of the season during which the first fertilizer applications were made, samples were collected of the leaves and the one and two-year branch growths, as it was considered that these would be best suited to indicate the response to the treatments. The bark in all cases was separated from the wood. The preliminary studies of this material have been limited to the untreated trees and those receiving the complete fertilizer both under sod and under tillage.

The purpose of determining the constituents present in the leaves was to ascertain if the analytical data of the leaves alone would furnish sufficient evidence of the condition of the trees. The results, on the percentage basis, have not as yet shown much promise that this can be accomplished. Furthermore, it is not

apparent that anything is to be gained in examining the two-year shoots if sufficient one-year material is available. The composition of the bark and wood, though actually quite different, is relatively of the same order. Table I gives the analytical results for the one-year shoots.

We consider that this experiment is hardly more than started and that it will be many years before we know what the real results will be. To us, one of the noteworthy results already secured is that two departments have been able to cooperate in this work for nearly 10 years without friction. Without this cooperation worthwhile progress can not be made in this kind of work.

The preliminary study of the chemical phases shows something of the rôle of nitrogen in the metabolism of the apple; but it also shows how much is still to be learned of the function of this necessary element.

In the one year wood from the trees grown in the rims the C/N ratios, in round numbers, in the order check-sod, check-tillage, N. P. K.-sod, N. P. K.-tillage are 276, 122, 71 and 86; and in the one year bark 49, 34, 32 and 26, respectively. The corresponding ratios in the leaves are 23, 19, 15 and 16.

The N/P_2O_5 ratios are also interesting. In the above order the ratios are: wood, 0.67, 1.09, 1.91 and 1.60; bark, 2.53, 2.60, 3.04, and 3.00. These figures seem to indicate that in this soil the check trees (both tillage and sod) are supplied with nitrogen at a lower rate than is required even for the utilization of the relatively small quantity of available phosphoric acid in the native soil. The C/N and N/P_2O_5 ratios thus give a valuable index as to the relative excess or deficiency of nitrogen and phosphoric acid.

No relationship between the potash content, either of the leaves, or wood, or bark and growth response, is apparent. In the case of these young trees growing in this Hagerstown silty loam soil, therefore, it would seem that enough potash is supplied to furnish this constituent at a rate sufficient to provide for normal growth without fertilization with this element even under conditions of extreme root crowding.

The first year's analyses of the material grown under the controlled conditions of this experiment show that the trees in the check row, both under sod and under tillage, have a nitrogen supply that is quite insufficient to utilize the stored carbohydrates for growth, as shown by the ratio of starch to total nitrogen, or of the "total" carbohydrates to nitrogen.

The first application of nitrogen seems to have stimulated a greater proportional branch elongation in the sod trees than in those under cultivation, but not enough total growth to overcome the check caused by the sod in 1924. At the same time there were indications that the average leaf on the sod-nitrated trees had a somewhat lower dry weight compared with similarly treated trees under tillage.

The response in fruit bud formation to the different treatments was striking. The row receiving a complete fertilizer produced

TABLE I—NITROGEN, CARBOHYDRATE AND MINERAL CONTENT OF ONE-YEAR GROWTH

IN PERCENTAGES CALCULATED ON THE DRY WEIGHT OF MATERIAL

Treatment	Description	Ash	Total Sugars	Starch	Acid Hydrolyzable Substances	Total Carbohydrates	Nitrogen	Ratio Total Carbohydrates	Ratio Starch	K^2O	P^2O^5	Ratio P^2O^5
									N			N
Sod, check........	Wood	1.64	2.18	10.19	23.13	41.50	0.150	276.6	67.93	0.185	0.223	1.48
Sod, check........	Bark	6.90	2.88	11.42	17.53	31.83	0.650	48.9	17.57	0.689	0.256	0.39
Tillage, check.....	Wood	1.38	2.10	8.48	21.45	32.04	0.261	122.7	32.49	0.243	0.239	0.91
Tillage, check.....	Bark	6.57	3.35	7.43	13.10	23.88	0.691	34.5	10.75	0.846	0.266	0.38
Sod, NPK........	Wood	1.72	1.63	7.42	24.47	33.52	0.472	71.0	15.72	0.250	0.246	0.52
Sod, NPK........	Bark	7.59	2.93	8.21	18.57	29.71	0.932	31.8	8.80	0.957	0.306	0.32
Tillage, NPK......	Wood	1.08	1.34	6.22	21.71	29.27	0.342	85.5	18.12	0.210	0.215	0.78
Tillage, NPK......	Bark	7.58	2.95	5.74	14.78	23.45	0.880	26.6	6.52	0.760	0.295	0.33

646 blossom clusters; the N. P. trees 477 clusters; the N. K. trees 115; the trees which received only nitrogen had 92, while the check row gave 6 blossom clusters. Nitrogen alone produced more blossoms under cultivation than under sod, while N. K. produced more under sod.

All these results throw little enough light on the original problem,—the physiology of the apple under different conditions—but there are other results which do give a more definite answer as to the value of the methods being used.

Has the use of the iron rims been of any value in this work? We think it has been of great value in that it has accentuated soil conditions and given results in a year which would have required at least a decade to produce under field conditions and might not have been obtained at all.

How long can trees be grown under these conditions? We expect that by the time they are eight years old many of these trees will have passed through a physiological cycle from infancy to decrepit old age and be ready to be discarded.

The very fact that the use of these rims accentuates conditions renders it very necessary to use extreme caution in drawing any conclusions from the results. Fortunately we have somewhat similar experiments under field conditions which have been under way for 18 years. In spite of this we will plant a field duplicate of this rim experiment this spring if we are successful in obtaining the asexually propagated roots for it and we will expect to repeat the work in the rims when the present trees go out. When the evidence from all these is collected we hope to have returns which will justify our very considerable expenditure of time and money.

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NITROGENOUS METABOLISM OF *PYRUS MALUS* L.

I. INFLUENCE OF TEMPERATURE OF DESICCATION ON WATER-SOLUBLE NITROGENOUS CONSTITUENTS AND SEPARATION OF WATER-SOLUBLE PROTEIN FROM NON-PROTEIN CONSTITUENTS[1]

WALTER THOMAS

Introduction

Notwithstanding the fact that nitrogen occupies a unique position in plant metabolism, our knowledge concerning the processes of absorption, synthesis or ultimate disposition of its compounds in the metabolic cycle of the higher plants is very limited.

The various groups of the complex combinations which nitrogen is capable of forming may have quite different physiological functions and, accordingly, as SPOEHR and MCGEE (**23**) have already emphasized, it is improbable that a rational conception of the function of nitrogen in the higher plants can ever be obtained without further studies of the physiological rôle of its various groups. Thus, a knowledge of the part these groups play and the extent to which their reactivity can be controlled are questions that must be solved before the problems of growth, reproduction and movement can be solved.

Owing to the very large number and complexity of the compounds which nitrogen forms, the separation and identification of its various groups or compounds are beset with difficulties; but the advances made in the chemistry of proteins have paved the way for an approach to investigations on nitrogen metabolism that were not available to the older workers in this field (**9**).

The possibilities which lie in the acquisition of greater knowledge of these weighty problems by the more modern method of approach are indicated by Chibnall's (**2**) studies on the distribution of nitrogen in the leaves of the Runner Bean (*Phaseolus vulgaris*) throughout a vegetative cycle;

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The cost of these investigations, forming a part of a cooperative project with the Department of Horticulture, has largely been defrayed by a grant from the Adams Fund for Agricultural Research.

but no investigations have been reported on the distribution or partition of nitrogen in the tissues of fruit trees throughout a cycle.

The present studies were undertaken for the purpose of obtaining data that might give an insight into the transformations taking place, as measured by the nitrogen fractions soluble in water, at the different vegetative periods throughout a year's cycle. When the possibilities and limitations of such studies are known it should then be possible to determine to what extent such information might be utilized, more especially in relation to the problems of growth and reproduction. The problem necessarily involves the simultaneous study of the validity of methods.

A. The effect of temperature of desiccation on the water-soluble nitrogenous constituents

As the nature of the problem necessitated the accumulation of samples for subsequent analyses, prevention of enzymatic activity as soon as possible after the samples are collected is essential. Preservation by means of alcohol is undesirable because of its precipitating effect on the proteins. Freezing also produces marked changes in some plants (**27**). The available evidence indicates that inactivation of proteolysis by dehydration by drying probably causes less changes in the tissues than any of the methods at present in use. The question of the optimum temperature at which autolytic changes are reduced to a minimum has received attention recently by Tottingham, Schulz and Lepkovsky (**27**) and by Link and Schulz (**17**). These papers and also a paper by Chibnall (**3**) contain a discussion (with references) of the temperatures adopted by previous investigators for the desiccation of the tissues of various plants. None of the earlier workers appear to have made any investigation into the effects of temperature on the composition of the plants under experiment.

Although Chibnall (**3**) found, in the case of the leaves of the Runner Bean, that low-temperature drying in a closed oven at 40–50° C. entailed protein autolysis, causing an increase in ammonium salts, asparagine and amino acids, the American investigators (**17, 27**), under the conditions adopted by them, found that the greatest changes produced either by rapid drying or freezing resulted from the coagulation of soluble proteins. Coagulation was greatest at high temperatures; at low temperatures (32–40° C.) both coagulation and proteolytic changes seemed to occur.

Experimental

The material used for this investigation was obtained from Stayman Winesap apple trees 15 years old growing in the College orchard. Samples of leaves and new shoot growth were collected in the early part of a July morning when active growth was in progress.

Temperatures adopted in this investigation

The experiences of previous investigators (**17, 27**) that very high and very low temperatures are to be avoided, minimum changes occurring at "intermediate" temperatures (50–70° C.), afford a sufficient basis for the selection of the three temperatures, 50, 60 and 70° C., for the determination of the optimum temperature causing minimum proteolytic changes in the water-soluble nitrogenous constituents of the species under investigation.

At 50 and 60° C. desiccation was carried out in ordinary Freas ventilated ovens. Drying at 70° C. was accomplished in wire bottom trays in a drying closet heated by steam coils. To further restrict the possibility of enzymatic activity the moisture content was reduced to 0.5 per cent. by transference to a vacuum oven for eight hours after the samples had been ground. The samples were preserved in glass stoppered sealed bottles. A month elapsed before analytical work was commenced on the dried samples.

Preparation of samples for analysis

The removal of the vacuole and colloidal cytoplasmic cell contents from the fresh leaves was readily accomplished by means of a Nixtamal mill (**19**) which readily disintegrates the cells of succulent plant materials after two or three passages through the mill. However, the disintegration of the cells of the refractory woody shoot growth could not be accomplished by this means. This was finally effected by the laborious process of alternate passage through an Enterprise mill and mincing by hand and afterwards macerating with quartz sand in the manner to be described later. The dried material was easily ground by a method previously described (**26**).

Method of extraction of the soluble nitrogenous constituents: Direct method versus Chibnall's procedure

Tottingham, Schulz and Lepkovsky (loc. cit.) found the direct extraction with water to be a satisfactory method for partitioning the products of the nitrogenous constituents of barberry and sugar beet leaves. Water enabled them to extract the greater part of the proteins exposed by crushing the cells of the fresh tissues, the direct method compared with Chibnall's (**4, 5**) giving somewhat higher values for total water-soluble nitrogen.

Comparison of the direct method with Chibnall's method by the writer on apple leaves indicated that only two-thirds as much protein N and non-protein N were found in the extracts obtained by the latter method. These results would indicate that the modifying action of the cytolytic agent (ether) in Chibnall's procedure is of greater influence than the post-mortem changes due to the "setting" of the cytoplasm into a gel (**6**).

All extractions were made by grinding 50 to 100 gm. of the material in a mortar with water (about 200–300 cc.) containing 20 cc. of 2 per cent. phenol. The brownish red extract was decanted on a filter of paper pulp, using a Buchner funnel, and the residue, after washing with water, again transferred to the mortar for further grinding. The process of filtering and grinding was repeated until microscopic examination showed that 85–90 per cent. of the cells were broken up. All samples were made up to a volume of 2,200 cc. and covered with a layer of toluene.

METHODS OF ANALYSIS

The methods of determining the various fractions will be given in detail in the third paper of this series in connection with the partition experiments.

TABLE I

TOTAL WATER-SOLUBLE N, NON-PROTEIN N, AMMONIA N AND AMIDE N IN THE LEAVES

a. AS PERCENTAGES OF THE DRY MATTER

SERIES NUMBER	TOTAL N	TOTAL WATER SOLUBLE N	NON-PROTEIN N	AMMONIA N	AMIDE N
	Per cent.	Per cent.	Per cent.	Per cent.	Per cent.
OA					
Fresh	1.80	0.130	0.089	None	0.014
Dried at 50°	1.82	0.120	0.090	None	0.016
Dried at 60°	1.82	0.120	0.092	....	....
Dried at 70°	1.83	0.109	0.098	None	0.016
18					
Fresh	1.86	0.146	0.120	0.003	0.013
Dried at 50°	1.86	0.144	0.124	0.006	0.017
Dried at 60°	1.85	0.144	0.123	0.006	0.017

b. AS PERCENTAGES OF THE TOTAL NITROGEN OF THE DRY MATTER

SERIES NUMBER	TOTAL N	TOTAL WATER SOLUBLE N	NON-PROTEIN N	AMMONIA N	AMIDE N
	Per cent.	Per cent.	Per cent.	Per cent.	Per cent.
OA					
Fresh	1.80	7.22	4.94	....	0.77
Dried at 50°	1.82	6.59	4.94	....	0.88
Dried at 60°	1.82	6.59	5.05	....	....
Dried at 70°	1.83	5.95	5.35	....	0.87
18					
Fresh	1.86	7.85	6.45	0.16	0.70
Dried at 50°	1.86	7.74	6.66	0.32	0.91
Dried at 60°	1.85	7.59	6.65	0.32	0.91

In the present experiments the effects of desiccation on the sap contents was followed by observation of the changes in the total water-soluble N, non-protein N, ammonia N and amide N.

The results obtained are given in tables I and II.

TABLE II

TOTAL WATER-SOLUBLE N, NON-PROTEIN N, AMMONIA N AND AMIDE N IN THE NEW GROWTH

a. AS PERCENTAGES OF THE DRY MATTER

SERIES NUMBER	TOTAL N	TOTAL WATER SOLUBLE N	NON-PROTEIN N	AMMONIA N	AMIDE N
	Per cent.	Per cent.	Per cent.	Per cent.	Per cent.
OB					
Fresh	0.715	0.163	0.099	0.003	0.009
Dried at 50°	0.715	0.155	0.110	0.004	0.014
Dried at 60°	0.711	0.155	0.104	....	....
Dried at 70°	0.710	0.137	0.103	0.004	0.011

b. AS PERCENTAGES OF THE TOTAL NITROGEN OF THE DRY MATTER

SERIES NUMBER	TOTAL N	TOTAL WATER SOLUBLE N	NON-PROTEIN N	AMMONIA N	AMIDE N
	Per cent.	Per cent.	Per cent.	Per cent.	Per cent.
OB					
Fresh	0.715	22.8	13.8	0.419	1.250
Dried at 50°	0.715	21.6	15.3	0.559	1.950
Dried at 60°	0.711	21.8	14.6	....	....
Dried at 70°	0.710	19.3	14.5	0.563	1.549

Discussion

These results, as also those of TOTTINGHAM and his co-workers (**27**), indicate that the higher the temperature (within limits) the greater becomes its influence on the coagulation of soluble proteins. In the case of the woody branch growth this appears to be accompanied by greater proteolytic changes at the lowest temperature (50° C.), a possible explanation of which is the longer time required for the desiccation of ligneous tissues.

It is apparent, however, that desiccation of the tissues of this plant species, which is low in water-soluble nitrogen, under the temperature conditions described, does not greatly change the proportions of total water-soluble N and non-protein N and that the changes in the ammonia N and amide N are not, except in the woody tissues, much greater than the error of determination. The optimum temperature appears to be around 60° C.

These findings and those of LINK and SCHULZ (**17**) on the barberry suggest that the proteolytic enzymes, due possibly to the different nature of the nitrogenous or carbohydrate constituents, may be relatively less active in plants low in water-soluble nitrogen than in those that are relatively high in these constituents. But whether, as suggested by LINK and SCHULZ, the differences observed between the high and low water-soluble nitrogen types of plants are due to the initial water content and the rate of its removal can, it seems to the writer, only be determined by further investigations in which plants low in the soluble cytoplasmic proteins are compared with those that are high (**7**). Until such questions are cleared up each plant tissue must represent a separate problem, in which case generalizations extended to all types of tissues are impossible.

B. THE SEPARATION OF THE PROTEINS FROM THE NON-PROTEIN CONSTITUENTS

Although, for the accurate determination of the amino nitrogen content, the separation of the proteins from the other nitrogenous constituents of the extracts of plant cells is by some investigators (**24**) considered unnecessary, others (**28**) have shown the advantage of doing so. The water-soluble protein in the plant species under investigation is, as we have seen, quite low; nevertheless, apart from the desirability of having some knowledge concerning its amount and fluctuations during the vegetative cycle, positive evidence was obtained that the removal of the soluble protein by the method to be described herein enabled more consistent results to be obtained in the estimation of the several non-protein fractions with which these metabolism investigations are mainly concerned.

The objective is to effect the separation with the removal of as little as possible of the intermediate products of protein degradation; but whether any reagent accomplishes the complete exclusion of all products which are only a little below the original proteins in their complexity from the protein-separates is doubtful.

NOTES ON THE REAGENTS USED IN THIS INVESTIGATION

Since STUTZER (**25**) proposed copper hydroxide for the removal of "peptides" from the "amide N," a number of reagents, such as methyl alcohol, acetic acid, trichloroacetic acid, phosphotungstic acid, tannic acid, and colloidal ferric hydroxide, have been used for the separation of the "protein" from the "non-protein" constituents, and, unfortunately, the evidence points to the conclusion that a reagent satisfactory for one kind of biological material may be unsuitable for another.

HART and BENTLEY (**13**) have shown that STUTZER's reagent may not, in all cases, effect a complete separation of all protein and polypeptide structures from amines and amides, for, although the copper salts of a number of di-, tri-, and tetra-peptides have been prepared, having the formula (peptide)$_1$:Cu (**16**), not all peptides may form insoluble copper salts under the conditions usually adopted for the precipitation. From the standpoint of the present investigations the chief objection to the use of copper salts is that under the conditions adopted they form insoluble copper compounds with certain amino acids.

BLISH (**1**), working on wheat flour extracts, following a suggestion by OSBORNE and LEAVENWORTH (**20**), obtained very nearly complete separation. He treated the extracts with 0.1 N NaOH followed by 0.1 N CuSO$_4$ until the latter was only in slight excess of the equivalent required. Although his precipitates were free from amino groups, evidence was presented that peptide linkings of unknown nature but of less complexity than true proteins were still present in small amounts in the filtrates.

Both acetic acid and trichloroacetic acid are used by a large number of investigators both in the animal and plant physiological fields. The use of the latter was proposed by GREENWALD (**11**) because of its easy solubility, its ready volatility and its non-solvent action on proteins when used in excess. It is claimed that it does not precipitate either the proteoses or the peptones (**12**), although the results obtained in this investigation are inexplicable on this basis.

Colloidal ferric hydroxide was first introduced by RONA and MICHAELIS (**22**), since which time it has received more favor as a precipitant for blood protein than possibly any other reagent. The evidence presented by CLEMENTI (**8**) and also by VAN SLYKE, VENOGRAD-VILLCHUR and LOSEE (**29**) indicates that this reagent precipitates none of the amino acids; the latter also found that it precipitates none of the intermediary products up to the proteoses and none of these except some of complexity but little below that of the original proteins. Moreover, since the present investigations were completed, FODOR and REIFENBERG (**10**) have shown that the proteins adsorbed by the ferric hydroxide gel can be recovered completely by eluating the residuum with NaOH.

Mercuric salts have the advantage that they remove all pigments, giving colorless solutions; but the excess of mercury is not conveniently and easily removed. Like copper they also precipitate polypeptides.

Experimental

The effects of four of these reagents on the nitrogenous constituents of the water extracts of the leaves of *Pyrus Malus* were examined. The technique of precipitating the soluble proteins with the various reagents was as follows:

Acetic acid and trichloroacetic acid.—The extract (2,000 cc.), after the addition of 10 cc. of 10 per cent. acetic acid or of 25 cc. of a 10 per cent. solution of trichloroacetic acid, was heated to boiling and boiled one to two minutes. The coagulum was separated by a fluted filter paper, washed with 100 cc. hot water, and made up to the original volume.

Copper hydroxide.—To the extract, measuring about 2,000 cc. in a 4 liter flask, was added 400 cc. of a freshly made-up solution of 0.2 N NaOH, followed by 400 cc. of 0.2 N $CuSO_4$. Ten cc. portions of the 0.7 N $CuSO_4$ were then added, and after each addition the extract was well shaken and the precipitate allowed to subside. The copper-protein precipitate was removed by filtration and washed with water.

Colloidal ferric hydroxide.—Merck's colloidal ferric hydroxide was used. The extracts were brought just to the boiling point, when an excess of the reagent, determined by examining the filtrates for freedom from proteins, is added. In the present experiments 5–6 cc. is sufficient. The extracts were then brought to the neutral point and the boiling continued for one or two minutes. In partition experiments of the nature to be described, it is necessary to remove the excess of iron by the addition of $MgSO_4$ or some other salt of high coagulating power. From 0.5 to 1.0 cc. of a saturated solution of this salt is sufficient to produce coagulation of the iron gel. The precipitate of protein-ferric hydroxide gel is separated by filtration, the precipitate washed, and the filtrate made up to the original volume.

DETERMINATION OF WATER-SOLUBLE PROTEIN NITROGEN

In all cases the protein N was determined by difference between the non-protein N and the total water-soluble N. The large fluted filter necessary for the filtering required too large a blank to enable the small amount of protein to be determined directly with any degree of accuracy.

In table III the mean of closely agreeing duplicate determinations is given.

TABLE III

COMPARISON OF PROTEIN N, NON-PROTEIN N AND AMINO N IN THE WATER EXTRACTS

SERIES NO. OC	NITROGEN IN THE DRY MATTER				
PRECIPITATING REAGENT	TOTAL N	TOTAL WATER SOLUBLE N	PROTEIN N	NON-PROTEIN N	AMINO N
	Per cent.	Per cent.	Per cent.	Per cent.	Per cent.
	1.83	....	....	....	....
Acetic acid	....	0.144	0.034	0.110	0.014
Trichloroacetic acid	....	0.146	0.034	0.112	0.018
Copper hydroxide	....	0.146	0.078	0.038	0.008
Colloidal ferric hydrate	....	0.142	0.022	0.120	0.024

QUALITATIVE TESTS ON THE FILTRATES FROM THE VARIOUS PRECIPITATING REAGENTS

The filtrates from the various reagents used for removing the proteins were subjected to the biuret, Millon and Adamkiewicz reactions. Such tests serve as a general guide, but caution must be used in drawing conclusions, for, as MOLISCH (**18**) has pointed out, the blue color of the biuret test is given also by certain carbohydrates and a number of aliphatic acids; hence, the test is not specific for proteins and the $-NH_2-CO-CO-NHR-$ groups. Neither can the Millon test be regarded as specific for the tyrosine and tryptophane groups of proteins, because it is also given by phenol, salicylic acid and other substances containing a monohydroxybenzene nucleus, upon the presence of which the test for proteins depends. The biuret test is best carried out by the method of KANTOR and GIES (**15**), who describe the preparation of a stable biuret reagent which is more sensitive than the classical method of application. The Adamkiewicz reaction was found in the present investigation to be more sensitive if the solution to be tested is concentrated almost to dryness *in vacuo*, followed by extraction with glacial acetic acid and concentrated HCl and not concentrated H_2SO_4 as usually recommended.

Some investigators (**10**) use SPIEGLER and ESSBACH's reactions to differentiate the derived from the non-derived proteins (*i.e.*, the simple and conjugate proteins free from their hydrolytic products). However, it is questionable (**14**) if these reagents are specific.

One of the difficulties encountered in applying such tests to plant extracts is the difficulty in removing all pigments present without also removing some of the products to be tested. The present tests were made on carbon black-treated extracts, which were concentrated *in vacuo* after filtering.

The results are conveniently shown in table IV.

TABLE IV

QUALITATIVE TESTS OF EXTRACTS FOR PROTEINS

REAGENT	FILTRATES	BIURET	ADAMKIEWICZ	MILLON
Copper salt	clear	negative	negative	slight positive
Acetic acid	turbid	positive	positive	positive
Trichloroacetic acid	turbid	positive	positive	negative
Colloidal ferric hydroxide	clear	positive	positive	positive

Qualitative tests, however, although devised for pure proteins and other decomposition products, may throw additional light on the results obtained by quantitative means and thus serve as an aid in interpretation.

Discussion

The quantitative results, table III, show that $CuSO_4$ and NaOH have removed more nitrogen from solution than the other reagents. This appears to be the result of the occlusion or formation of insoluble copper-amino-acid compounds (*e.g.*, asparagine) as well as to the complete removal of the specific peptides occurring in these extracts together with products intermediate between these and the true proteins. The negative biuret reactions in the copper filtrates give further confirmation of this, for a positive test is first encountered in certain tetrapeptides and is the more intense the greater the length of the polypeptide chain (**21**). This same test shows that the other reagents have not removed peptides and the intermediate products. The colloidal ferric hydroxide gel contains the lowest amount of nitrogen and the highest quantity of amino N. The presence of proteose N in the filtrates from the colloidal iron treatment may be objectionable from the standpoint of its influence on the determination of amide N, because of the possible error in the determination of amide N in the process of hydrolyzing the extracts. This was not investigated further because the amounts present are so small that the effect on the analytical results would be insignificant. The evidence to be presented in the third paper of this series shows clearly that all filtrates contain the CONH groupings.

Colloidal ferric hydroxide, then, is admirably suited for the separation of true proteins from solution because: (1) It is more convenient and expeditious; (2) it permits all the amino acids to go through, occluding and precipitating none; (3) it effects sharp separation of true protein from its decomposition products; and (4) the adsorbed proteins can be recovered quantitatively from the residuum.

Summary

Desiccation of the leaves and new wood growth from apple trees at 50°, 60° or 70° C. has no effect on the total nitrogen; but relatively small decreases in the soluble cytoplasmic proteins, and increases in the non-protein constituents, occur through drying. Considering the effect of temperature on both coagulation and proteolysis, it would appear that a temperature of 60° C. is the optimum for the desiccation of the tissues of this species, which belongs to the type of plants that are low in water-soluble protein nitrogen.

Colloidal ferric hydroxide has many advantages over the reagents in general use for the separation of the simple and conjugate proteins from their hydrolytic products. Reasons for this choice are given.

The writer takes this opportunity of expressing his thanks to Professors J. B. Hill and L. O. Overholts for microscopic examination of some of the extracted materials.

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NITROGENOUS METABOLISM OF *PYRUS MALUS* L.

II. THE DISTRIBUTION OF NITROGEN IN THE INSOLUBLE CYTOPLASMIC PROTEINS[1]

Walter Thomas

Before undertaking the experimental work on the distribution, throughout a year's cycle, of the soluble nitrogenous constituents of the leaves and branch growth, which will be presented in the third paper, it was considered of interest to obtain information respecting the nature of the insoluble leaf proteins and to ascertain what fluctuations, if any, occur during development, in the distribution of its nitrogen groups (*i.e.*, its amino acid make-up). An additional motive for a closer study of this insoluble portion was afforded by the relationships which Nightingale (**8**) seemed to find between his alcohol insoluble nitrogen fraction, and carbohydrates, in the vegetative and reproductive processes of the tomato plant (*Lycopersicum esculentum*).

Sufficient knowledge is not yet available to enable these insoluble proteins to be isolated in a state of purity, but Van Slyke (**11, 12**) has devised a method for determining the distribution of nitrogen in the various groups of the protein molecule which has furnished means of classifying different proteins by their amino acid make-up. This method was, however, devised for pure proteins and their hydrolytic products (**1, 2, 3**), but as Morrow and Gortner (**7**) have pointed out, the extension of the method may be made to impure proteinaceous substances when due precautions are taken and no attempt is made to assign any part of the nitrogen to any specific amino acid.

Experimental

Samples of leaves were collected, from a 15-year-old Stayman Winesap tree growing in sod, at three critical physiological periods, *viz.* (1) at the early period of bud formation (May 13), (2) at the stage of active maximum growth (July 22), and (3) at the period of chlorophyl degeneration, *i.e.*, of declining metabolic activity (Nov. 11).

Five grams of each residuum, dried *in vacuo*, remaining after the extraction with water as described in the preceding paper and consisting, therefore, of the insoluble cytoplasmic proteins and the cellulose material of the cell wall, together with such non-protein nitrogenous materials as fats, lecithins, chlorophyl, and the purine and pyrimidine bases, were extracted with alcohol followed by ether for the removal of fats, chlorophyl and lecithins, and afterwards digested with 0.5 per cent. HCl (**10**) to hydrolyze the interfering carbohydrates (**5**). After filtering and washing with hot water, each residuum, consisting of the impure proteins, was hydrolyzed with 20 per cent. HCl for 48 hours on a sand bath, with the usual precautions, to insure comparable results (**4**). The small amount of protein N in the filtrate from the 0.5 per cent. HCl hydrolysis was separated and hydrolyzed after the process described by Hamilton, Nevens and Grindley (**5**). After the removal of the HCl by distillation *in vacuo*, the two hydrolysates were combined. The residue was dissolved in 100 cc. of water and a slight excess of MgO added. The process of determining the nitrogen fractions was then, with slight modifications, similar to that described by Morrow and Gortner (**7**).

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The analytical data are presented in table I.

TABLE I

The insoluble cytoplasmic proteins

Distribution of N as percentage of total nitrogen

Nitrogen fraction	Series no. 3		Series no. 20		Series no. 33	
	(a)	(b)	(a)	(b)	(a)	(b)
Ammonia N .... (A)	15.50	15.20	14.80	14.20	16.40	16.00
Insoluble humin N .... (B)	7.82	8.00	7.75	7.70	9.00	8.80
Humin N precipitated by $Ca(OH)_2$ ... (C)	0.23	0.20	0.24	0.27	0.24	0.29
Total humin N .... (A + B)	8.05	8.15	7.99	7.97	9.24	9.19
Total basic N .... (D)	20.62	20.55	21.80	21.70	22.05	20.30
Basic N set free as NH_3 by 50% KOH (arginine) .... (E)	5.80	lost	5.92	6.00	6.34	6.15
Basic N not set free as NH_3 by 50% KOH .... (D – E)	14.82	....	15.88	15.70	15.71	14.15
Amino N of bases .... (F)	5.25	....		....		....
Non-amino N of bases .... (D – F)	15.37	....		....		....
N in filtrate from bases .... (G)	53.10	52.80	54.85	54.91	55.00	54.20
Amino N in filtrate from bases .... (H)	51.30	51.00	53.20	....	53.00	....
Non-amino N in filtrate from bases .... (G – H)	1.80	1.80	1.65	....	2.00	....
Total N obtained	97.27	....	98.72	99.44	102.69	99.80

In series no. 33 (a) fats, lecithins, chlorophyl, etc., were removed by previous extraction with alcohol and then with ether. This would contain a small amount of nitrogen, but the total amount as compared with the protein N present is small.

Discussion and conclusion

Is it justifiable to draw from the constancy of the distribution of nitrogen the conclusion that, although the leaf proteins vary quantitatively throughout their cycle, they do not vary qualitatively? In other words, do these results indicate that a single protein exists in the leaves or do they contain a mixture of several? Osborne (**9**) believes that even if several proteins occur in these leaves they may be so nearly alike in their amino acid make-up that changes in their relative proportions can not be established by methods at present available. He is of the opinion that, though the above results do not give *absolute proof*, perhaps, that a single protein is present in these leaves, they do *indicate* that this is the case.

Even if these "insoluble" proteins could be isolated in the pure condition so that not only the percentage of amino acids but also their optical properties, molecular weight and elementary composition could be determined, the similarity of all these would not constitute indisputable evidence that the proteins were identical. If marked differences occurred in the amino acid content there would be little question as to the existence of a mixture of substances of unlike chemical entities; but these results, until new methods of study become available that enable the question of the true chemical entity of the proteins to be determined, do indicate that little, if any, qualitative change in the nature of the insoluble leaf protein has taken place during its development.

From this standpoint, then, it is clear that any relationship to plant performance that may be discoverable between the insoluble proteins and carbohydrates must be due to quantitative and not qualitative differences in the nature of the insoluble proteins.

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NITROGENOUS METABOLISM OF *PYRUS MALUS* L.

III. THE PARTITION OF NITROGEN IN THE LEAVES, ONE AND TWO YEAR BRANCH GROWTH AND NON-BEARING SPURS THROUGHOUT A YEAR'S CYCLE*

Walter Thomas

(WITH FOUR FIGURES)

Introduction

Osborne and his colleagues (**23**) in discussing the status of plant chemistry state: "It is true that we know a multitude of products derived from plants, and we know much of the chemistry of these, but this knowledge consists mostly of isolated facts which contribute comparatively little to a knowledge of the chemical make-up of the plant as a whole." This situation is due to the fact that the largest contributions to our knowledge of the compounds formed by plants have emanated from investigators in the chemical laboratories of large pharmaceutical manufacturing establishments who have vigorously prosecuted research in this direction for the purpose chiefly of isolating products of interest and value in therapeutics. Such investigations are not concerned with the energetics or dynamics of the plant, *i. e.*, with the functions and fate of the compounds isolated, in their relation to the response of the plant to the internal conditions existing during successive stages of their life cycle, by whatever means these may have been produced.

The theories of Liebig, though shown later to be false, spurred the early agricultural chemists and physiologists to carry out a prodigious amount of work on the quantitative changes of the various elements—ions—absorbed by the plant throughout the principal vegetative periods. Much of this work is abortive because the methods then used do not conform to modern standards of accuracy. The rapid advances during the last two decades in the chemistry of proteins and the products of their hydrolysis necessitates the investigation of the function of nitrogen with the application of this newer knowledge.

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Scope and limitations of this investigation

The investigation of the dynamic systems produced by the large number of chemical reactions which have given rise to the various nitrogen compounds in the plant, and of the function of the individual groups in metabolism, is a laborious task involving much observational and analytical work. A quantitative measure of the changes undergone by the various nitrogen groups throughout a year's cycle is outlined in this paper. But what specific rôle is played by each in the metabolic cycle and in what manner their activity can be controlled are problems for further investigation. In the next paper it will be shown in what manner fertilizer application of an inorganic salt like $NaNO_3$ can modify the seasonal changes in some of the principal nitrogen fractions.

It is doubtful if, in the present state of our knowledge, all the data accumulated in these investigations can be interpreted. A great deal of information and insight, however, can be obtained with respect to the transformations taking place and also to the relationships of the various fractions to one another. The bearing of the results presented in this paper to practical horticulture has been presented in a previous report (**37**). It is of interest to note that no relationship could be discovered between either temperature or sunshine or rainfall or humidity and any of the nitrogen fractions.

The content of soluble nitrogenous constituents of the cells from aerial parts of *Pyrus malus* is relatively low (**35**); thus, only seven to ten per cent. (depending upon the season of the year) of the total nitrogen of the leaves is soluble in water. This is the case whether the soluble cell products are obtained by means of cytolytic agents or by direct extraction with water. In these investigations preference is given to the latter method (**35**). Since nothing was to be gained by any further investigation of the soluble cytoplasmic proteins (**36**), the next logical procedure appeared to be an examination of the metabolically active or reactive phases of the products of the nitrogen transformations. These are measured by quantitative determinations, during a year's cycle, of the fluctuations of the various groups of nitrogen fractions soluble in water. The relationships of these fractions to one another and to any external factor or factors have also been investigated.

Catalysers in growth and reproduction

The classical investigations of Emmerling (**10**) had indicated the relationship of amino-acids and amides to the synthesis of proteins, but their rôle in growth had not been stressed until MacDougal and Spoehr and McGee postulated that amino-acids and amides were the principal fractions influencing the rate of growth by functioning as catalytic agents, either by increasing the hydration capacity of the pentosan-protein colloids, as suggested by the former (**21**), or by accelerating the rate of carbohydrate katabolism, as indicated by the latter (**33**).

Although the investigation *in vitro* (even in a crude and rough fashion) of the course of definite reaction types, with the hope of discovering analogies between them and the complicated processes in the cell, may furnish valuable clues, nevertheless the extension and generalization of dogmatic interpretations and conclusions from experiments like MacDougal's, carried out *in vitro* to processes *in vivo*, must be made with considerable reservation. Moreover, in Spoehr and McGee's experiments dealing with the effect of feeding glycocoll on the stimulation of the rate of respiration and carbohydrate consumption, the plants and also excised leaves were subjected to more complete shading than would occur under normal growth conditions; hence, the conclusions drawn are hardly applicable to the normal processes of the plant. Again, the behavior of nitrogenous compounds as catalytic agents is not peculiar to amino-acids, for Dakin (**9**) found that not only amino-acids but also peptone could be used to catalyse certain condensations at low temperatures, and Knoevenagel (cited by Dakin, *loc. cit.* p. 50) found that even organic bases (diethylamine, piperidine, aniline, and other bases) could be used to bring about a number of syntheses, at low temperatures, such as the union of aldehydes and peptones, and substances containing the $-CO-CH_2.CO-$ group. Since the other nitrogen fractions, *viz.*, ammonia, amides, peptides, basic compounds and also those unclassified nitrogen compounds that do not respond to the tests in any scheme thus far developed for partitioning the complex nitrogenous compounds of plants—the so-called "rest" N fraction—are shown in these experiments to take part in the nitrogen transformation during the vegetative cycle, the specific rôle of each fraction in relation to the synthesis and degradation of proteins, and, therefore, in the growth and reproduction processes must also be considered. The present investigations indicate that these "rest" N compounds may play as important a rôle as the amino-acids. The action of catalyzers is attributed to their capacity to exist in different states of oxidation. Nitrogen, copper, manganese and iron, for this reason, are especially active catalytic agents. Cook (**6**) has given evidence from which one may deduce that iron and copper are the prime oxidation catalysts in cell metabolism. The stressing of the importance of amino-acids has had the unfortunate effect of leading some to believe that determination of these acids may indicate or be a measure of the vigor of growth; but this hypothesis is not borne out in the present investigations. It is possible, therefore, that the "rest" N compounds and possibly other fractions may play a rôle equally as important as amino-acids.

Materials and methods

Eliminating variables.—Since the different parts of a fruit tree appear to behave as independent units (**1, 19**) and because variations of composition occur from the tip to the basal part of the shoot (**39**), the elimination of all controllable variables is essential. For this reason, in all experimental work involving analyses, fertilizers should be applied uniformly around the root system and care exercised to sample the tree uniformly. It is obvious, also, that if comparisons are to be made between two or more trees they should be of the same age and variety, growing in a homogenous soil. These principles were adhered to in these investigations.

Difficulties inherent to analytical investigations in perennial woody plants.—Harvey and Murneek (**14**) have discussed the analytical difficulties of experimental work with woody perennials such as fruit trees in comparison with herbaceous annuals like the tomato. Reference has been made to one of the disadvantages, *viz.*, the behavior of the different parts of fruit trees as independent units; another disadvantage lies in the relatively small percentage of active tissue present, in consequence of which special refinement in analytical methods is essential. Still another difficulty that is not encountered in herbaceous plants lies in the relative alteration in the proportions of wood to bark with age. The ratio of wood to bark varies from the apex to the base of the branch in the sense that it increases with the age of the growth. A determination of the *absolute* changes in magnitude of any group or constituent throughout the vegetative cycle is, therefore, precluded. However, relative fluctuations and especially the direction of these changes, as influenced by external factors, can be studied. The difficulty of the wood-to-bark ratio may, in part, be overcome by separating the bark from the wood and analyzing each separately; but even this method is not free from criticism, for during the interval required to separate the bark from the wood enzymatic changes can take place that might seriously affect the results. Analyses have been made both with and without separation in these investigations, with the result that no advantage is derived from such a separation. Moreover, the interpretation of results is not changed by the method of procedure in this respect, because, although the bark is higher than the wood in total nitrogen, it is lower in most of the water-soluble fractions; consequently, when the curves for amino-acids and amides, for example, are drawn for the wood and the bark separately throughout the cycle, the relative increase in mass of wood to bark is counterbalanced by the somewhat higher amounts of these fractions in the wood compared with the bark.

A Stayman Winesap tree, 15 years old, in the "off" bearing year, of known history and performance, growing in sod in the College Experimental Orchard, was used in this investigation. It received an application of 5 lbs. $NaNO_3$ on April 7, 1923. The desirability of restricting the work to one tree is obvious as a means of eliminating variability. Although a composite sample of a number of trees, provided they were of the same variety and age, if available, would give larger samples, thus facilitating analytical work, the possible advantages to be gained by this procedure would be counterbalanced by the disadvantages attendant upon the inability, in a preliminary investigation, to focus attention upon the behavior of one plant.

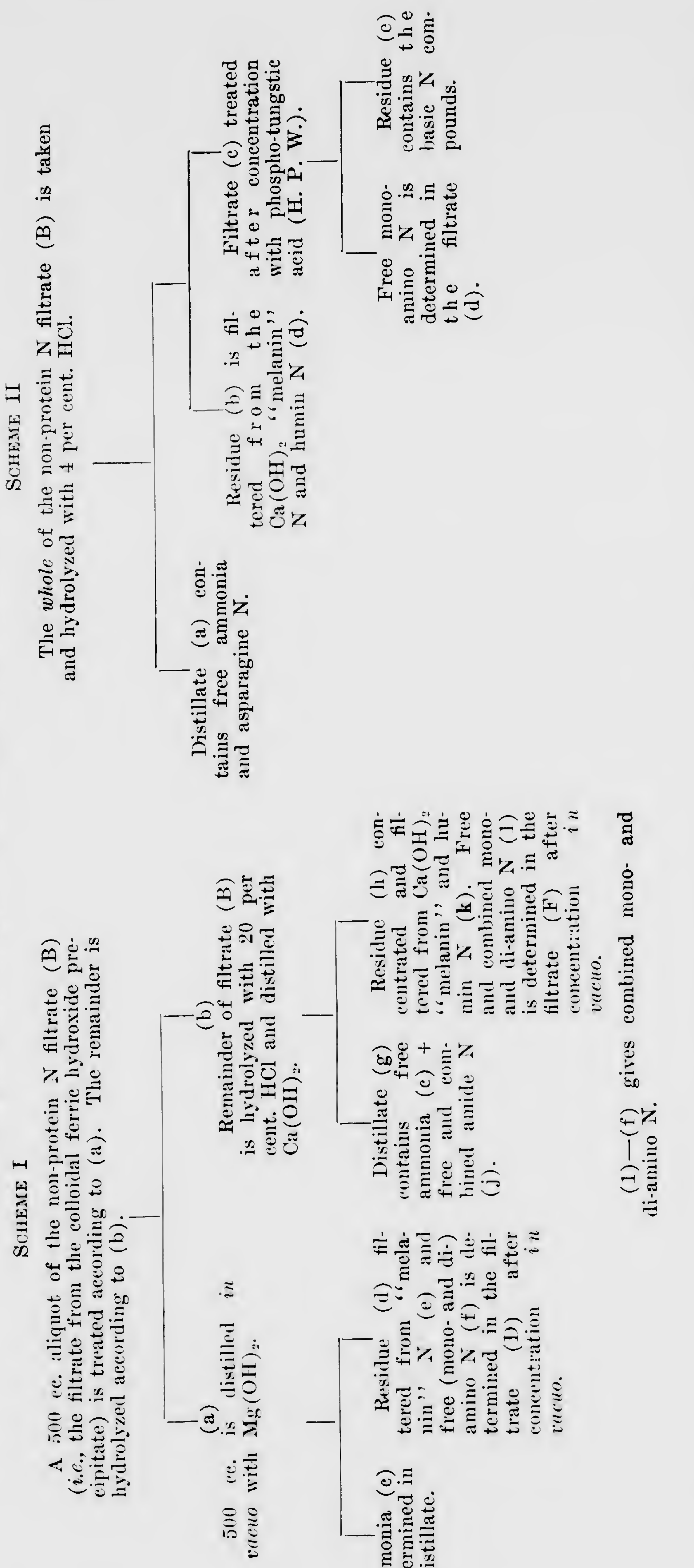
Samples of leaves, non-bearing spurs, and branches of three different ages (one year, two years, and three to seven years) were taken at eleven dates between June 18, 1923, and June 11, 1924. The sampling was done at times when the meteorological (rainfall and humidity) conditions had been favorable for three consecutive days preceding the taking of the samples. The diurnal variations which occur in the tissues of the apple tree were found to be small compared with the seasonal changes. They were avoided by always taking the samples at the same time of the day, in the early morning. Although a small decrease occurred in the total nitrogen at night, no changes could be observed in the total water-soluble products during this period.

Fractionation methods

The method of extraction has already been described (**35**). Some investigators effect the extraction of nitrogenous constituents by means of boiling water. This method is, however, objectionable not only because a certain degree of peptonization of proteins may take place, but many nucleins are decomposed by boiling water with formation of hypoxanthine, etc. (**30**). Water at 40° C. was used for the extraction in this investigation.

Any method of separating the nitrogen fractions will necessarily be based upon the work of the army of investigators who have contributed to our knowledge of the analysis of proteins and their hydrolytic products. Among the contemporary workers who have sought to apply the nitrogen distribution methods to heterogeneous compounds of plant origin should be mentioned Chibnall, Williams, and the Illinois group

THE FRACTIONATION METHODS ADOPTED IN THESE INVESTIGATIONS



SCHEME I

A 500 cc. aliquot of the non-protein N filtrate (B) (*i.e.*, the filtrate from the colloidal ferric hydroxide precipitate) is treated according to (a). The remainder is hydrolyzed according to (b).

- (a) 500 cc. is distilled *in vacuo* with $Mg(OH)_2$.
  - Ammonia (c) is determined in the distillate.
  - Residue (d) filtered from "melanin" N (e) and free (mono- and di-) amino N (f) is determined in the filtrate (D) after concentration *in vacuo*.
- (b) Remainder of filtrate (B) is hydrolyzed with 20 per cent. HCl and distilled with $Ca(OH)_2$.
  - Distillate (g) contains free ammonia (c) + free and combined amide N (j).
  - Residue (h) concentrated and filtered from $Ca(OH)_2$ "melanin" and humin N (k). Free and combined mono- and di-amino N (l) is determined in the filtrate (F) after concentration *in vacuo*.

(1)—(f) gives combined mono- and di-amino N.

SCHEME II

The *whole* of the non-protein N filtrate (B) is taken and hydrolyzed with 4 per cent. HCl.

- Distillate (a) contains free ammonia and asparagine N.
- Residue (b) is filtered from the $Ca(OH)_2$ "melanin" N and humin N (d).
- Filtrate (c) treated after concentration with phospho-tungstic acid (H. P. W.).
  - Free mono-amino N is determined in the filtrate (d).
  - Residue (e) contains the basic N compounds.

(HAMILTON, GRINDLEY, and colleagues), who have contributed numerous papers in this field.

Two different methods of separation have been used by the writer as a basis for the characterization of the various non-protein fractions. These two methods are, for clarity, represented here diagrammatically.

The two schemes differ with respect to the rigor of the hydrolysis, resulting in a differentiation with respect to (*a*) the distribution of the mono-amino- and the di-amino-acids, and (*b*) of the substances determined as amide N. Most of the complex peptide linkings would be decomposed by the stronger hydrolysis with 20 per cent. HCl, so that whereas the amide N in fractionation scheme I includes the combined as well as the free $-CO-NH_2$ groups, the mild hydrolysis adopted in fractionation scheme II gives as amide N only asparagine and other similarly constituted acid amides, *i. e.*, those having the $-CO-NH_2$ group free. Again in scheme I the free amino (both mono- and di-) and also the combined amino N (both mono- and di-) are determined *separately*, whereas in scheme II compounds containing the combined di-amino N groups are precipitated with the bases. The latter fractionation method will, therefore, give only the free mono-amino N.

A group of not very clearly defined nitrogenous compounds, *viz.*, the so-called "humin" and "melanin" fractions are separated in both fractionation methods. The origin of the "humin" N from the hydrolysis of proteins has been studied by GORTNER *et al* (**11, 12, 13, 16**). The term "melanin" N is used by the writer to denote the compounds precipitated by $Ca(OH)_2$ or $Mg(OH)_2$. It represents the nitrogen belonging to the acid pigment-like bodies found in the sap of plants. MORROW and GORTNER (**22**) believe that this nitrogen may also be derived from the calcium salts of the purine and pyrimidine bases. This, however, is questionable, for the barium salts of the pyrimidines are quite soluble as are also some of the alkali earth salts of the purines like thiobromine sodium.

As already mentioned, there remains in both fractionation schemes a group of nitrogen compounds, usually designated as "rest" N or "other" N, which do not respond to the tests in any scheme thus far developed for determining the partition products. This residual N cannot represent identical nitrogen compounds in the two methods. Thus, in scheme I the "rest" N will represent the difference between the total non-protein N and the sum of the ammonia, free (mono- and di-) amino, combined (mono- and di-) amino, free and combined amide and also "humin" nitrogen; while in scheme II, based on the HAUSMANN (**15**) separation of the mono- and di-amino acids by phosphotungstic acid (H. P. W.), the "rest" N will represent the difference between the total non-protein N and the sum of the ammonia, free mono-amino N, asparagine N (amide N) (**28**).

basic N and "humin" N. These basic nitrogen compounds comprise the derivatives of trimethyl-amine, hygrine, adenine, choline, betaine, stachydrine; the purine bases; and also the basic di-amino compounds like arginine, histidine, lysine and cystine.

The first fractionation scheme then determines both mono- and di-amino acids, but the bases and di-amino acids are removed by phosphotungstic acid in the second fractionation method, and which, accordingly, estimates only the mono-amino acids. It will be shown in a later paper that the "rest" N compounds of scheme II are higher throughout the cycle than those of scheme I. This indicates (*a*) that the peptide combinations present *in these filtrates* are not precipitated by phosphotungstic acid, though doubtless, as found by VICKERY (**43**), some polypeptides may be precipitated by phosphotungstic acid; or (*b*) that the combined mono- and di-amino N compounds (*i. e.*, compounds containing peptide linkages) are present in the non-protein fraction in much larger amounts than the basic nitrogen; or (*c*) that a portion of the "rest" N fraction has undergone decomposition during the more vigorous hydrolyses adopted in scheme I. Thus, CHIBNALL (**3**) found in his starvation experiments that hydrolysis with strong acids completely decomposed the "rest" N compounds, half their nitrogen appearing as ammonia N and about one-third as bases.

Without going into details respecting the nature of the substances precipitated by phosphotungstic acid, from the literature of the subject (**18**, **24**, **31**), it is certain that a variable product is obtained, depending to some extent on the concentration of the solution, and *even in the case of the decomposition products of pure proteins*, other decomposition products besides the purines and the di-amino acids, histidine, arginine, and lysine appear to be precipitated by phosphotungsic acid, according to conditions. Probably leucine and tyrosine may also be precipitated (**31**). KIESEL (**18**) under the conditions of precipitation used by him found both phenylalanine and aspartic acid in the phosphotungstic acid precipitate. These results are undoubtedly due to the working conditions. Since the phosphotungstates of some amino acids are relatively insoluble, as a precautionary measure, it is always desirable to dilute to a large volume before attempting separation of the bases (**42**).

The first fractionation scheme was adopted in the 1923-24 experiments which are described in this paper and the second scheme in the experiments to be described in a later paper. Although undoubtedly superior to the second scheme, from the standpoint of accuracy, the first method possesses the disadvantage of being time-consuming, owing to the additional separations required.

DETAILS OF ANALYTICAL PROCEDURE

Attention is called at the outset to the fact that nitric N was found only at one period—just as the buds are opening. This point has been described fully elsewhere (**38**).

(*a*) AMMONIA N.—This includes the ammonia of ammonium salts and is usually described in the literature as "free" ammonia for the purpose of differentiating between this ammonia and that formed by the hydrolysis of amides. Inasmuch as the protein-iron gel (**35**) is more readily filtered if heated, and inasmuch as a slight decomposition of peptides occurs under certain not well-defined conditions, it is advisable to determine ammonia on a 200 cc. aliquot of the *original extract* denoted as (A), before removal of the water-soluble proteins. The determination is best carried out in the apparatus and by the method described by VAN SLYKE (**40**). It is only fair to state here, since it is not generally known, that the method for ammonia N is essentially that described by LONGI (**20**), who distilled at a temperature of 38-40° C. with $Mg(OH)_2$. He noted that neither urea nor asparagine were decomposed under these conditions. The writer prefers solid CaO (**26**) to MgO for the reason described later.

(*b*) FREE MONO- AND DI-AMINO N.—The removal of the "free" ammonia must be first carried out as described under (*a*) *from a 500 cc. aliquot of the filtrate* previously freed from soluble proteins in the manner already described (**35**). This filtrate freed from proteins is denoted as (B.) CaO is now better substituted for MgO. It is advantageous to use only a very slight excess of *solid* CaO, the exact amount being determined by a preliminary titration (**26**). With this precaution little or no frothing occurs in the VAN SLYKE apparatus during the determination of amino N. Pure CaO is recommended in preference to MgO because in the experience of the writer the former occludes or absorbs more of the pigments to which most of the frothing can be attributed. No success was obtained with secondary octyl (caprylic) alcohol (even twice redistilled, B. P. 131° C.) which has been recommended for the purpose of reducing the surface tension and, consequently, the frothing (**41**). The blanks from this reagent, due presumably to the evolution of some gas that is not absorbed by the alkaline permanganate mixture, were found too high to justify its use in the determination of the relatively small amount of amino N measured in these experiments.

A small and somewhat variable quantity of *nitrogenous matter* will always be *precipitated*, occluded or absorbed by the CaO. This is "melanin" N and is removed by filtration and washed with hot water; the filtrate, which is denoted as (C), is acidified with acetic acid and afterwards concentrated under a pressure of 15 mm. to about 15 cc. using a CLAISSEN flask (**40**). In this concentrated solution a further precipitate containing Si, Al, Ca, Na and K salts usually separates out. This precipitate is removed by filtration and the filtrate, which is denoted as (D), made up to a definite volume prior to its introduction into the VAN SLYKE micro-apparatus (**40, 41**).

The writer has confirmed SPOEHR and MCGEE's observations (**32**) with respect to the alleged interference of carbohydrates in the nitrous acid method (cited by SPOEHR and MCGEE, *loc. cit.* p. 34, footnote). The determination of the amino N in aspartic acid was not altered by the presence of glucose or fructose.

(*c*) FREE AND COMBINED AMIDE N.—A definite volume of concentrated HCl sufficient to give a 20 per cent. solution is now added to the remainder of the original filtrate (B) containing the non-protein nitrogenous constituents, *i.e.*, to the extract remaining after the removal of the aliquots for the determination of free and combined amino N as under (*b*) above. The mixture is then hydrolyzed for twelve hours with the usual precautions; after the removal of the HCl *in vacuo* (**40**) the residue is taken up in water and distilled *in vacuo* with a slight excess of solid CaO. A titration of the distillate gives both the "free" ammonia N and the "free and combined" amide N. The difference between the titration values for ammonia obtained as described under (*a*) and (*c*) gives the "free and combined" amide N. The solution still remaining in the flask is denoted as (E).

(*d*) COMBINED MONO- AND DI-AMINO N.—The solution (E) left in the flask from the determination of the free and combined amide N (*c*) is filtered from the CaO "melanin" nitrogenous material, washed, and the combined filtrates and washings (F) acidified with acetic acid and concentrated *in vacuo* to a volume of about 15 cc. A determination of the amino N in this gives the "free" and also the "combined" amino N. The latter, which is indicative of the complexity of the peptide linkings, is determined by the difference in the values obtained in this determination and that given under (*b*).

Experimental results

The results of the analyses by the foregoing methods are given in the following tables. Table I gives the percentages of imbibitional and total water in branches of different ages, and in the short spurs and leaves. Table II shows the water-soluble nitrogenous constituents of the leaves, branches of different ages, and short spurs. The partition of the non-protein fraction is shown for various collecting dates in percentages of the green weight and dry weight.

TABLE I

THE PERCENTAGES OF IMBIBITIONAL AND TOTAL WATER

ONE-YEAR BRANCH GROWTH

SERIES NO.	COLLECTING DATE	FRESH WEIGHT	DRY WEIGHT	IMBIBITIONAL WATER	TOTAL WATER
		gm.	gm.	Per cent.	Per cent.
1	June 18, 1923	55.0	26.3	52.1	57.6
7a	July 16, 1923	102.0	50.7	50.3	55.3
15	August 17, 1923	41.8	20.5	51.0	56.5
27	September 17, 1923	129.0	60.0	53.5	56.7
34	October 15, 1923	104.0	52.5	50.0	54.7
46	November 20, 1923	125.0	66.2	47.0	51.8
56	April 12, 1924	120.0	62.0	48.3	54.0
64	April 21, 1924	80.0	40.0	50.0	54.0
80	April 28, 1924	69.0	34.3	50.7	54.0
98	May 13, 1924	90.0	40.0	55.6	57.9
118	May 22, 1924	57.0	23.0	59.6	61.0
143	June 11, 1924	56.0	23.8	57.5	58.0

TWO-YEAR BRANCH GROWTH

SERIES NO.	COLLECTING DATE	FRESH WEIGHT	DRY WEIGHT	IMBIBITIONAL WATER	TOTAL WATER
		gm.	gm.	Per cent.	Per cent.
03	June 18, 1923	75.0	35.4	52.8	54.2
7	July 16, 1923	150.0	75.0	50.0	54.0
19	August 17, 1923	69.5	33.8	52.0	54.5
31	September 17, 1923	167.0	87.0	47.9	53.7
38	October 15, 1923	155.0	85.0	45.2	50.7
47	November 20, 1923	161.0	87.0	44.7	49.8
57	April 12, 1924	65.0	35.1	46.0	48.0
65	April 21, 1924	82.0	45.0	45.1	51.1
97	April 28, 1924	125.0	67.0	46.4	52.4
119	May 22, 1924	132.0	65.0	50.8	54.8
147	June 11, 1924	140.0	70.0	50.0	53.8

OLDER GROWTH

SERIES NO.	COLLECTING DATE	FRESH WEIGHT	DRY WEIGHT	IMBIBITIONAL WATER	TOTAL WATER
		gm.	gm.	Per cent.	Per cent.
4	June 18, 1923	210.0	110.4	47.4	52.3
8a	July 16, 1923	230.0	118.4	48.4	52.3
14a	August 17, 1923	199.1	101.0	49.3	51.6
32	September 17, 1923	174.0	98.0	48.0	50.2
39	October 15, 1923	632.0	297.0	47.0	50.1
49	November 20, 1923	455.0	244.0	47.0	50.2
58	April 12, 1924	367.0	202.2	44.9	50.6
66	April 21, 1924	165.0	89.0	46.1	49.5
82	April 28, 1924	239.0	133.0	44.3	51.7
99	May 13, 1924	196.0	100.0	48.9	53.2
121	May 22, 1924	183.0	96.0	47.5	53.1
148	June 11, 1924	419.0	236.0	43.7	52.4
148a		127.0	58.0	54.3	

SHORT SPURS

SERIES NO.	COLLECTING DATE	FRESH WEIGHT	DRY WEIGHT	IMBIBITIONAL WATER	TOTAL WATER
		gm.	gm.	Per cent.	Per cent.
5	June 18, 1923	31.0	15.1	51.3	57.0
7	July 16, 1923	35.0	16.8	52.0	55.3
17	August 17, 1923	26.5	11.4	47.0	53.0
29	September 17, 1923	37.0	20.2	47.5	51.2
37	October 15, 1923	121.0	65.2	46.3	51.3
48	November 20, 1923	72.0	31.5	44.0	48.8
63	April 12, 1924	58.0	30.0	48.3	54.0
69	April 21, 1924	54.0	26.5	51.9	57.5
83	April 28, 1924	38.0	19.0	50.0	58.7
96	May 13, 1924	15.0	8.0	46.7	50.8
122	May 22, 1924	22.0	9.0	49.1	lost
149	June 11, 1924	2.5	1.9	24.0	46.0
150		5.5	2.7	50.9	

LEAVES

SERIES NO.	COLLECTING DATE	FRESH WEIGHT	DRY WEIGHT	IMBIBITIONAL WATER	TOTAL WATER
		gm.	gm.	Per cent.	Per cent.
01 001	June 18, 1923	38.0	17.2	54.8	57.0
6 6a	July 16, 1923	242.0	111.2	54.1	63.0
18 20	August 17, 1923	127.0	51.8	59.8	65.5
28 30	September 17, 1923	406.0	185.1	52.4	59.0
35 36	October 15, 1923	516.0	245.0	52.9	60.5
50	November 20, 1923	95.0	48.4	49.1	55.2
100	May 13, 1924	47.0	13.0	72.3	75.0
119a 123	May 22, 1924	72.0	lost	lost	lost
144	June 11, 1924	50.2	18.6	62.5	66.5
205	August 17, 1924	150.0	58.2	61.2	65.0

ACCURACY AND LIMITS OF ERROR

The errors of sampling could not be determined on account of the necessity for conserving the material, but the determination of the accuracy and limits of error of the analytical data has been made by expressing, as a percentage of the mean, the differences obtained in certain duplicate analyses. These are given in table III.

The total N shows a maximum difference of 0.58 per cent., the water-soluble N of 1.50 per cent., and the "free" mono- and di-amino N of 5.29 per cent. The experimental error in the "free" amino N is higher than that of the total nitrogen and total water-soluble N, because of the smaller amount of nitrogen determined.

Since the amide N is determined from the difference between the total NH_3 before and after hydrolysis, this determination will carry the errors of the ammonia determination also. It shows a maximum difference of 7.15 per cent. From the foregoing it is clear that all fluctuations in the nitrogen fractions can be regarded as significant.

Discussion and conclusions

(a) TOTAL NITROGEN

In general, the total nitrogen of the leaves (fig. 1) follows the course found for this species by other investigators (**2, 17**); these changes will, therefore, not be elaborated upon here, except to point out that the change

TABLE II

Amount of water-soluble nitrogenous substances present in the leaves, 1923 branch growth, 1922 branch growth, short spurs, and the partition of the non-protein portion expressed as percentages of fresh and dry weights, respectively

Leaves

Series no.	Collecting date	Total N	Protein N	Total water-soluble N	Total non-protein N	NH_3 N	Humin N	Free amino N	Combined amino N	Amide N	Residual N
				As percentages of fresh weight of material							
4A	June 18, 1923	0.719	0.0047	0.0606	0.0559	0.0013	0.0112	0.0162	0.0127	0.0094	0.0051
6	July 16, 1923	0.691	0.0030	0.0510	0.0482	0.0017	0.0087	0.0104	0.0126	0.0081	0.0066
20	August 17, 1923	0.641	0.0027	0.0430	0.0403	0.0024	0.0091	0.0070	0.0119	0.0024	0.0075
28	September 17, 1923	0.715	0.0038	0.0570	0.0532	0.0059	0.0119	0.0156	0.0176	0.0016	0.0006
35	October 15, 1923	0.646	0.0019	0.0422	0.0405	None	0.0022	0.0120	0.0135	0.0086	0.0040
50	November 20, 1923	0.585	0.0009	0.0280	0.0271	None	0.0013	0.0034	0.0027	0.0106	0.0091
100	May 13, 1924	0.877	0.0110	0.0820	0.0710	....	....	....	....	....	....
144	June 11, 1924	0.671	0.0060	0.0410	0.0350	....	....	....	....	....	....
				As percentages of dry weight of material							
4A	June 18, 1923	1.656	0.0110	0.1410	0.1300	0.0032	0.0261	0.0376	0.0300	0.0219	0.0120
6	July 16, 1923	1.869	0.0100	0.1370	0.1270	0.0046	0.0270	0.0281	0.0345	0.0177	0.0180
20	August 17, 1923	1.860	0.0076	0.1227	0.1151	0.0070	0.0250	0.0200	0.0341	0.0070	0.0220
28	September 17, 1923	1.746	0.0095	0.1425	0.1350	0.0147	0.0298	0.0390	0.0440	0.0044	0.0010
35	October 15, 1923	1.617	0.0048	0.1055	0.1013	None	0.0055	0.0300	0.0330	0.0215	0.0103
50	November 20, 1923	1.570	0.0020	0.0622	0.0600	None	0.0028	0.0075	0.0060	0.0239	0.0200
100	May 13, 1924	3.512	0.0440	0.3280	0.2840	....	....	....	....	....	....
144	June 11, 1924	2.000	0.0530	0.1206	0.0676	....	....	....	....	....	....

TABLE II—(*Continued*)

Amount of water-soluble nitrogenous substances present in the leaves, 1923 branch growth, 1922 branch growth, short spurs, and the partition of the non-protein portion expressed as percentages of fresh and dry weights, respectively

1923 branch growth

Series no.	Collecting date	Total N	Protein N	Total water-soluble N	Total non-protein N	NH_3 N	Humin N	Free amino N	Combined amino N	Amide N	Residual N
				As percentages of fresh weight of material							
1	June 18, 1923	0.269	0.0086	0.0507	0.0421	0.0012	0.0130	0.0125	None	0.0077	0.0077
5	July 16, 1923	0.324	0.0248	0.0739	0.0490	0.0014	0.0246	0.0175	None	0.0033	0.0022
15	August 17, 1923	0.288	0.0119	0.0717	0.0602	0.0092	0.0098	0.0185	0.0016	0.0057	0.0114
27	September 17, 1923	0.233	0.0025	0.0246	0.0221	0.0007	0.0051	0.0050	Trace	0.0052	0.0060
34	October 15, 1923	0.297	0.0066	0.0337	0.0271	0.0011	0.0039	0.0055	0.0057	0.0101	0.0008
46	November 20, 1923	0.328	0.0079	0.0451	0.0372	0.0020	0.0079	0.0079	None	0.0060	0.0134
56	April 12, 1924	0.272	0.0091	0.0268	0.0177	None	0.0023	0.0039	None	0.0088	0.0025
64	April 21, 1924	0.305	0.0142	0.0414	0.0262	None	0.0081	0.0105	None	0.0048	0.0012
80	April 28, 1924	0.304	0.0121	0.0576	0.0483	0.0031	0.0184	0.0114	0.0061	0.0054	0.0037
98	May 13, 1924	0.244	0.0071	0.0385	0.0280	0.0016	0.0026	0.0088	....	0.0075	0.0075
118	May 22, 1924	0.238	0.0027	0.0230	0.0203	0.0013	0.0023	0.0072	0.0013	0.0068	0.0013
144 144A	June 11, 1924	0.249	0.0056	0.0302	0.0245	0.0009	0.0047	0.0099	0.0024	0.0052	0.0015
				As percentages of dry weight of material							
1	June 18, 1923	0.372	0.0200	0.1180	0.0980	0.0028	0.0302	0.0290	None	0.0180	0.0180
5	July 16, 1923	0.326	0.0552	0.1642	0.1090	0.0031	0.0546	0.0390	....	0.0073	0.0050
15	August 17, 1923	0.352	0.0261	0.1630	0.1369	0.0209	0.0223	0.0420	0.0037	0.0210	0.0260
27	September 17, 1923	0.367	0.0060	0.0600	0.0540	0.0018	0.0124	0.0121	Trace	0.0130	0.0146
34	October 15, 1923	0.381	0.0146	0.0750	0.0604	0.0024	0.0086	0.0123	0.0126	0.0225	0.0017
46	November 20, 1923	0.467	0.0165	0.0939	0.0774	0.0041	0.0164	0.0165	None	0.0124	0.0280
56	April 12, 1924	0.419	0.0198	0.0582	0.0384	None	0.0050	0.0088	None	0.0191	0.0054
64	April 21, 1924	0.380	0.0332	0.0900	0.0568	None	0.0177	0.0206	None	0.0105	0.0080
80	April 28, 1924	0.347	0.0262	0.1252	0.1050	0.0068	0.0400	0.0248	0.0132	0.0117	0.0084
98	May 13, 1924	0.315	0.0239	0.0874	0.0637	0.0037	0.0060	0.0200	....	0.0170	0.0170
118	May 22, 1924	0.340	0.0060	0.0512	0.0452	0.0028	0.0050	0.0160	0.0030	0.0150	0.0029
144 144A	June 11, 1924	0.398	0.0120	0.0642	0.0522	0.0020	0.0100	0.0210	0.0050	0.0110	0.0032

TABLE II—(*Continued*)

AMOUNT OF WATER-SOLUBLE NITROGENOUS SUBSTANCES PRESENT IN THE LEAVES, 1923 BRANCH GROWTH, 1922 BRANCH GROWTH, SHORT SPURS, AND THE PARTITION OF THE NON-PROTEIN PORTION EXPRESSED AS PERCENTAGES OF FRESH AND DRY WEIGHTS, RESPECTIVELY

1922 BRANCH GROWTH

SERIES NO.	COLLECTING DATE	TOTAL N	PROTEIN N	TOTAL WATER-SOLUBLE N	TOTAL NON-PROTEIN N	NH_3 N	HUMIN N	FREE AMINO N	COMBINED AMINO N	AMIDE N	RESIDUAL N
				As percentages of fresh weight of material							
03	June 18, 1923	0.230	0.0110	0.0321	0.0211	0.0009	0.0055	0.0059	None	0.0055	0.0033
8	July 16, 1923	0.293	0.0179	0.0621	0.0442	0.0014	0.0224	0.0087	0.0067	0.0030	0.0020
19	August 17, 1923	0.221	0.0139	0.0389	0.0249	0.0040	0.0019	0.0049	0.0045	0.0046	0.0050
31	September 17, 1923	0.211	0.0047	0.0162	0.0113	None	0.0022	0.0025	Trace	0.0051	0.0014
38	October 15, 1923	0.240	0.0089	0.0261	0.0172	0.0012	0.0027	0.0054	0.0012	0.0089	0.0035
47	November 20, 1923	0.273	0.0131	0.0466	0.0335	None	0.0035	0.0080	0.0034	0.0060	0.0075
				As percentages of dry weight of material							
03	June 18, 1923	0.719	0.0240	0.0700	0.0460	0.0020	0.0120	0.0130	None	0.0120	0.0070
8	July 16, 1923	0.691	0.0390	0.1350	0.0960	0.0030	0.0488	0.0190	0.0146	0.0066	0.0040
19	August 17, 1923	0.641	0.0300	0.0836	0.0536	0.0086	0.0042	0.0106	0.0096	0.0100	0.0106
31	September 17, 1923	0.715	0.0100	0.0350	0.0253	None	0.0048	0.0065	Trace	0.0110	0.0030
38	October 15, 1923	0.646	0.0180	0.0530	0.0350	0.0024	0.0055	0.0110	0.0025	0.0180	0.0070
47	November 20, 1923	0.585	0.0260	0.0928	0.0668	None	0.0070	0.0160	0.0068	0.0120	0.0150

TABLE II—(*Concluded*)

AMOUNT OF WATER-SOLUBLE NITROGENOUS SUBSTANCES PRESENT IN THE LEAVES, 1923 BRANCH GROWTH, 1922 BRANCH GROWTH, SHORT SPURS, AND THE PARTITION OF THE NON-PROTEIN PORTION EXPRESSED AS PERCENTAGES OF FRESH AND DRY WEIGHTS, RESPECTIVELY

SHORT SPURS

SERIES NO.	COLLECTING DATE	TOTAL N	PROTEIN N	TOTAL WATER-SOLUBLE N	TOTAL NON-PROTEIN N	NH_3 N	HUMIN N	FREE AMINO N	COMBINED AMINO N	AMIDE N	RESIDUAL N
				As percentages of fresh weight of material							
2	June 18, 1923	0.372	0.0140	0.0355	0.0215	0.0013	0.0032	0.0047	None	0.0086	0.0037
7	July 16, 1923	0.326	0.0226	0.0502	0.0274	0.0019	0.0033	0.0104	None	0.0108	0.0009
17	August 17, 1923	0.352	0.0111	0.0959	0.0848	0.0230	0.0030	0.0199	0.0290	0.0029	0.0066
29	September 17, 1923	0.367	0.0098	0.0335	0.0237	0.0029	0.0036	0.0079	None	None	0.0093
36	October 15, 1923	0.381	0.0102	0.0510	0.0407	0.0041	0.0104	0.0147	None	0.0080	0.0034
48	November 20, 1923	0.467	0.0051	0.0446	0.0394	None	0.0102	0.0107	0.0037	0.0012	0.0134
				As percentages of dry weight of material							
2	June 18, 1923	0.844	0.0325	0.0825	0.0500	0.0030	0.0073	0.0110	None	0.0200	0.0087
7	July 16, 1923	0.726	0.0505	0.1115	0.0610	0.0042	0.0077	0.0231	None	0.0240	0.0020
17	August 17, 1923	0.752	0.0236	0.2040	0.1804	0.0489	0.0063	0.0423	0.0618	0.0061	0.0140
29	September 17, 1923	0.749	0.0200	0.0684	0.0484	0.0060	0.0073	0.0161	None	None	0.0190
36	October 15, 1923	0.780	0.0209	0.1040	0.0831	0.0084	0.0213	0.0300	None	0.0164	0.0070
48	November 20, 1923	0.916	0.0099	0.0857	0.0758	None	0.0197	0.0207	0.0072	0.0024	0.0258

TABLE III

DIFFERENCES OBTAINED IN CERTAIN DUPLICATE DETERMINATIONS AS PERCENTAGE OF THE MEAN

SERIES NO.	TOTAL N	TOTAL WATER-SOLUBLE N	MONO- AND DI-AMINO N	AMIDE N
6	0.58	1.20	5.20	6.00
15	0.44	1.40	3.40	3.10
19	0.50	0.35	1.80	2.00
28	0.32	0.00	4.00	7.15
34	0.00	0.50	0.25	2.30
48	0.18	1.50	3.40	0.80
98	0.32	0.75	3.80	1.50

in direction observed in September has occurred at approximately the same period every year the experiments were in progress. It is probably due to the diminished requirement of the branch growths for the nitrogen synthesized by the leaves.

THE EVACUATION OF NITROGEN FROM THE LEAVES IN THE FALL.—This so-called "Rückwanderung" or autumnal evacuation of nitrogen from the leaves to the branches in the fall has been asserted and as vigorously denied (**8**). Much of the difficulty in interpretation, however, can be attributed to the fact that in many instances attempts have been made to draw conclusions from analysis of the leaves only, omitting analysis of the perennial parts. Both COMBES (**4, 5**) and RIPPEL (**27**) have recently reported an extensive study of this phenomenon in young oak and beech trees. According to the former, the migration of nitrogenous substances commences as soon as the leaves turn yellow, slowly at first and then more rapidly. This is not in agreement with the results of RIPPEL (*loc. cit.*) nor of the writer; these latter clearly indicate that the evacuation of nitrogen in the leaves is not initiated suddenly at the onset of yellowing but much earlier. If the decrease commenced just as soon as yellowing occurred it might be argued that, since the nitrogen present in the leaves depends upon the difference between the amount of protein synthesized and that utilized in growth and respiration, and that, inasmuch as protein synthesis decreases in the autumn owing to the degeneration of the chloroplasts, this affords an explanation of the migration provided removal goes on at the normal rate. However, since there is sufficient evidence (*loc. cit.*) to indicate that the decrease in nitrogen may occur before degeneration of the chloroplasts occurs, this migration cannot be due to the same causes as that which occurs normally during the life of the tree, but must be distinct from the normal process. Admittedly, it is difficult to understand. SCHERTZ (**29**) has shown that a draft on the nitrogen of the leaf by the younger tissues leads to a decomposition of nitrogen compounds of the leaf including proteins, phospho-lipines and chlorophyll, the decomposition of the latter producing mottling. With the loss of chlorophyll accompanying autumnal coloration, the yellow pigments increase at its expense due to the combination of the phytol that is split off from the chlorophyll with sugars to form carotin and xanthophyll (cited by SCHERTZ, *loc. cit.* p. 124). There can be no doubt, however, that whatever the cause, the present investigations confirm the existence of this migration phenomenon. The concurrent increase in nitrogen during this period in the short spurs, one year, two year and older branch growth,

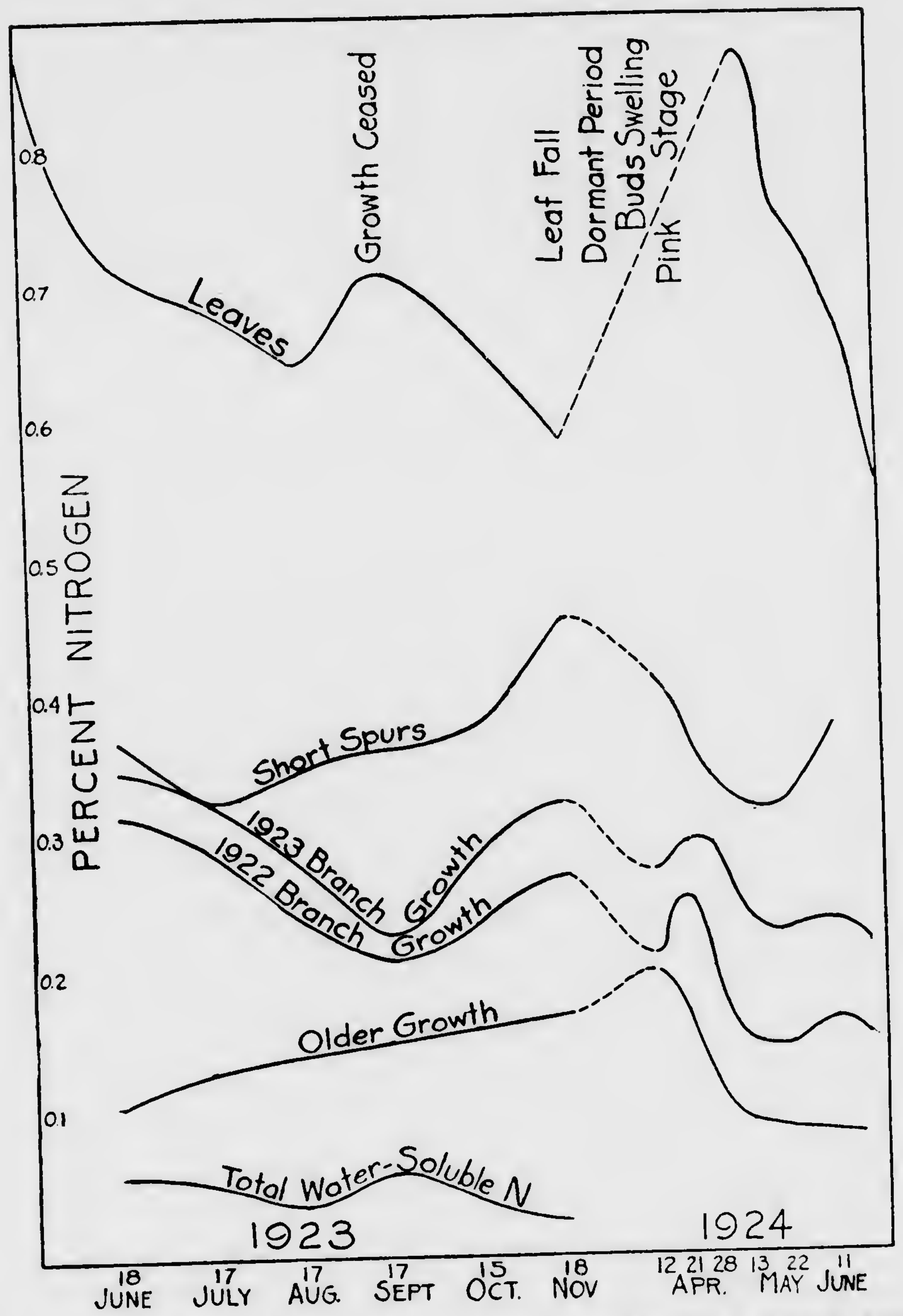


FIG. 1. Total nitrogen of the leaves, short non-bearing spurs, one-year, two-year and older branch growth as percentages of the fresh weight. June, 1923, to June, 1924.

which results from the evacuation from the leaves can readily be seen from an inspection of the analyses and fig. 1. The actual increase is probably greater than that shown if one allows for the relative increase of wood to bark. When the phenomenon of autumnal migration of nitrogen is considered in relation to the great demand of the shoots in the spring, as discussed above, this whole process may be looked upon as an expression of nature's method of conservation in the use of nitrogen.

THE PERIOD OF VEGETATIVE GROWTH.—Many investigators (9) have observed that a large decrease of nitrogen occurs in the older parts during the initiation of vegetative growth. Fig. 1 shows that during the period between the beginning of vegetative growth and the blossoming of the tree the loss of nitrogen from the branches is very large. The development of the buds and the formation of the new leaves are dependent upon the "reserve" proteins accumulated in the branches; a great expenditure of nitrogen compounds is thus required for growth of these parts. In some of the older tissues over half of the available stored nitrogen supply is given up to the young shoots, leaving a deficiency of nitrogen in these organs until this is supplied by the activity of the roots later in the spring.

A consideration of composite curves discussed elsewhere (**37**), showing the sum of the percentages of nitrogen in the leaves and in the one to seven year old branch growths, indicates that after *active* growth has ceased (third week in June) a condition of nitrogen equilibrium exists in the perennial parts until fall migration of nitrogen from the leaves commences.

CHANGES DURING THE DORMANT PERIOD.—The so-called dormant period is obviously accompanied by certain metabolic changes, for during this period with a mean daily temperature of 33.5° F. (the mean maximum being 35.5° F. and the mean minimum 20.2° F.) the total nitrogen in the short nonbearing spurs, one-year and two-year wood, decreased 11, 15 and 20 per cent., respectively.

During the commencement of the period of sap flow the nitrogen increased nearly 10 per cent. in the one-year wood and 18 per cent. in the two-year wood, and then during the rapid development of the shoot the nitrogen decreases rapidly in all parts due to the withdrawal of nitrogen stored in the phloem, as already stated. Compared with the quantities found when the tree entered the rest period, this amounts to a decrease of 32 per cent., 29 per cent., 21 per cent. and 22 per cent., respectively, in the short spurs, one-year branch growth, two-year branch growth, and older growth.

The form in which this decrease takes place cannot be measured by the changes in the water-soluble fractions taken at two periods only, because these are too small to account for the decrease involved. Apparently, there must be a continual degradation of the insoluble cytoplasmic proteins and

a transference to the older perennial parts throughout the whole dormant period.

(b) PARTITION OF NITROGEN IN THE LEAVES

In the leaves, the fluctuations (see figures 2 and 3) of the water-soluble, the non-protein, and also of the free and combined amino-nitrogen fractions vary inversely to their course in the branch growth throughout the whole cycle. The amide N does not appear to follow this or any other definite relationship. This inverse relationship in the leaves and branches between the groups mentioned is difficult to interpret because, although it is possible to express the data from the leaves on both an absolute and a percentage basis, the results on the branch growth cannot be so stated unless the entire tree is taken for analysis and this is impracticable in experiments of this type. Fig. 3 shows that the total water-soluble, the free (mono- and diamino), and also the free and combined amide nitrogen parallel the total nitrogen of the leaves throughout the cycle; the "rest" N usually varies inversely to them.

Although the amide N (free and combined) and also the "rest" N decrease in the fall during the period of migration of nitrogen from the leaves to the branches, the other fractions, *e.g.*, the total water-soluble, the

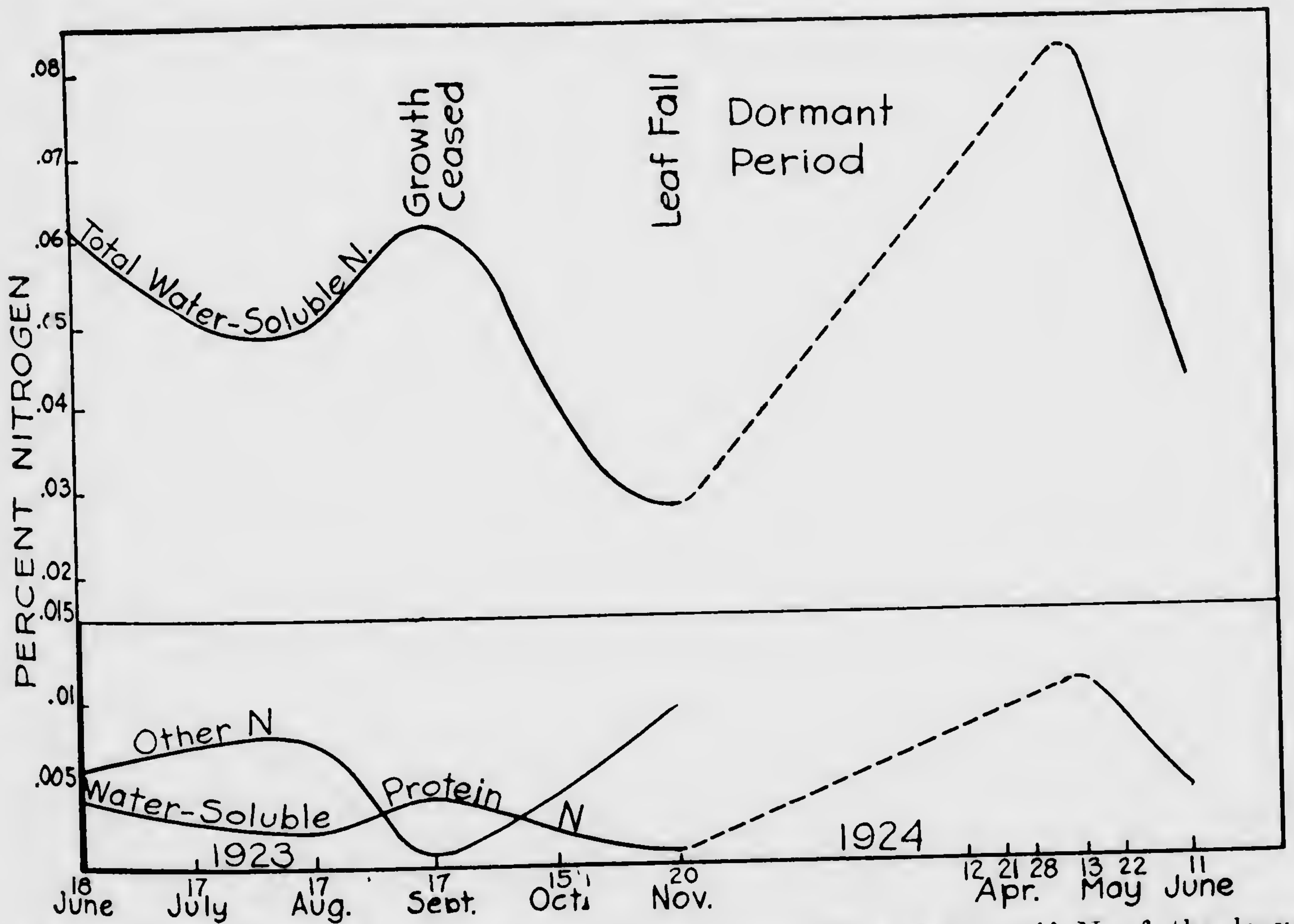


FIG. 2. Total water-soluble N, water-soluble protein N, and "rest" N of the leaves as percentages of the fresh weight.

non-protein, and the free (mono- and di-) amino-nitrogen decrease rapidly. The significance of this, to which reference will be made again, indicates that the "rest" N may have a close relation to protein degradation in the leaves (**3**). It is possible that nitrogen is translocated in the form of these "rest" N compounds. The water-soluble nitrogen is low throughout the cycle and varies inversely to the "rest" N. The significance of this is apparent.

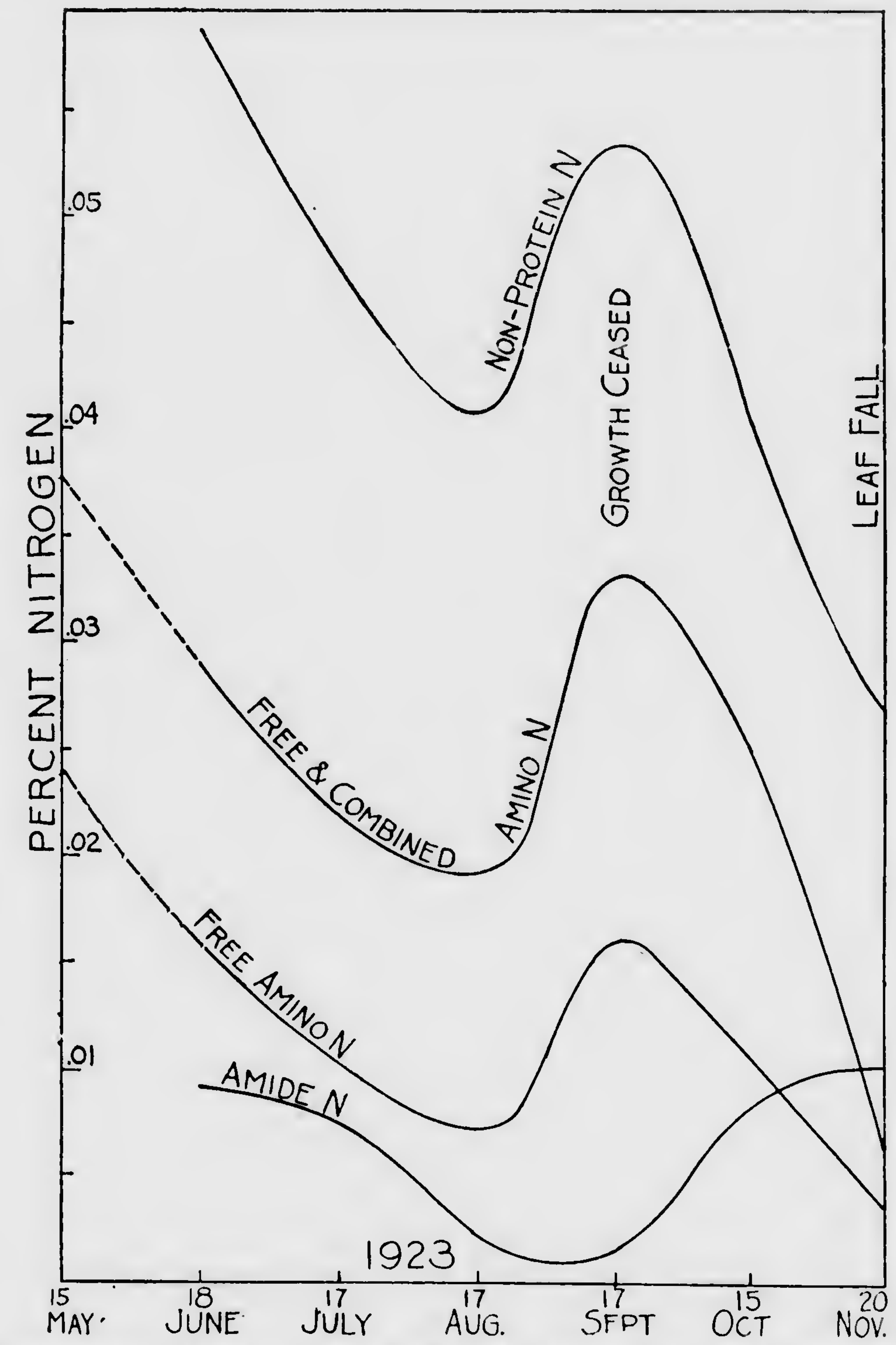


FIG. 3. The non-protein N, free and combined amino N, free amino N and amide N of the leaves as percentages of the fresh weight.

(c) PARTITION OF NITROGEN IN THE BRANCH GROWTH

The decrease in total nitrogen in the one- and two-year branch growth during the period of mobilization of reserve proteins (bud swelling) is accompanied by a rapid rise in the total water-soluble non-protein nitrogen, in the free amino, and also in the "rest" nitrogen fractions, the amide N

decreasing during this period (fig. 4). Possibly the increase in the amino N at this time may indicate that translocation in the flowing sap takes place during this period as amino-acids. That the transportation of the reserve material to the tissues during the germination of wheat seedlings takes place in large measure as free amino-acids has been shown by PETTIBONE and KENNEDY (**25**) and is thus analogous to the manner in which the proteins of the blood stream are transported to the tissues of animals. However, the fact that the "rest" N also parallels the amino N in magnitude and direction at this period indicates the importance of the rôle that these unknown fractions may also play. Both CHIBNALL and PRIANISCHNIKOW (see ref. **43**, p. 660) affirm that asparagine is the form in which nitrogen is translocated from one part of the plant to another, but, as

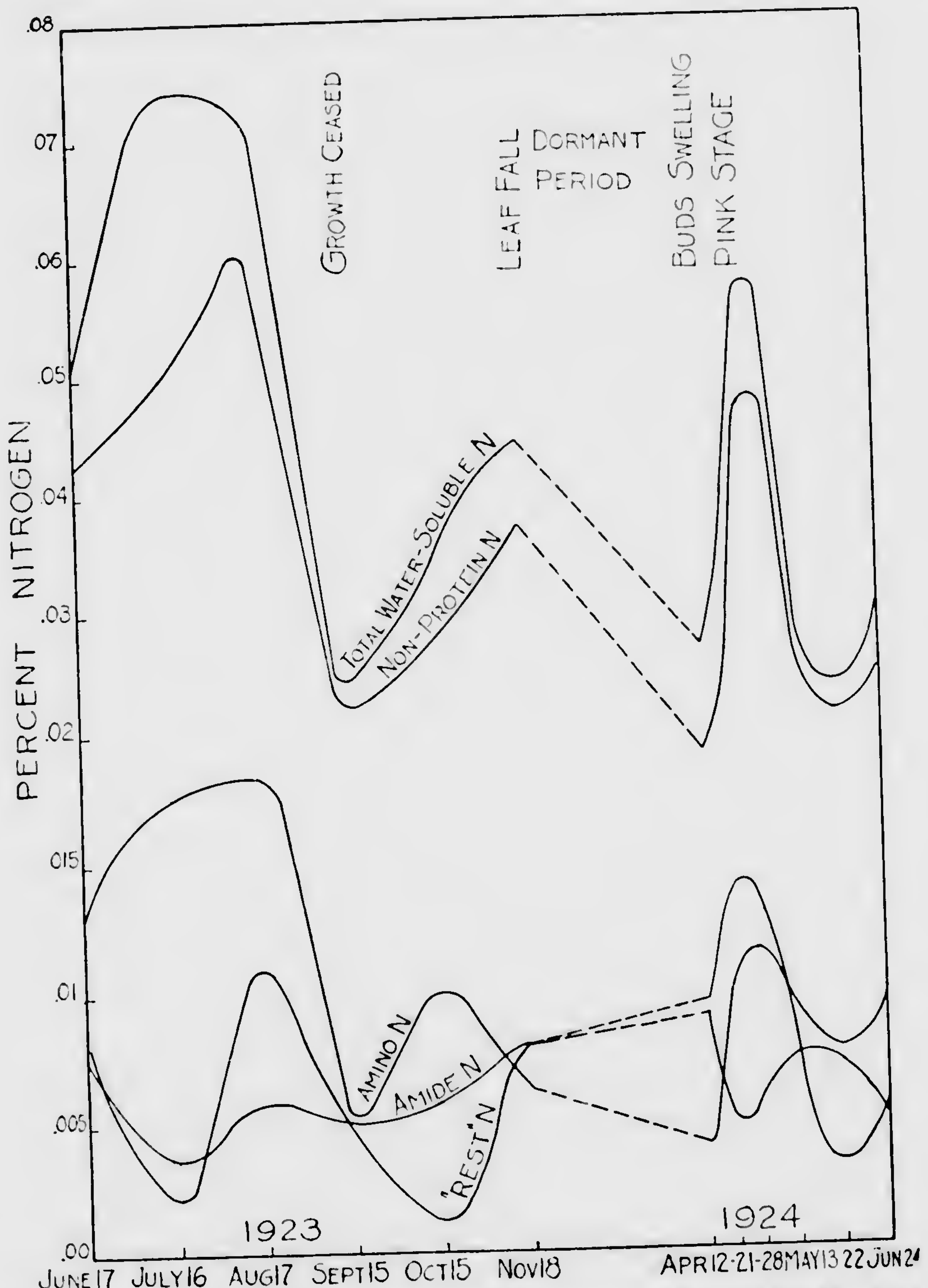


FIG. 4. Total water-soluble N, non-protein N, amino N, amide N, and "rest" N of one-year branch growth as percentages of the fresh weight.

pointed out by Vickery (**43**), the presence of a number of other amino-acids found by him in appreciable amounts in alfalfa sap may indicate that the translocating processes are not so simple as that indicated by Chibnall and Prianischnikow. It is expected that some additional light will be thrown on this subject later in the present series of investigation on *Pyrus malus*.

The sharp decrease in total nitrogen occurring in the one- and two-year branch growth and non-bearing spurs, between the beginning of vegetative growth and the period of blossoming, is accompanied by a rapid decrease in the total water-soluble, non-protein, and free amino nitrogen, but a gradual increase in the amide N. This seems to indicate that the material for the development of flowers was being drawn rapidly from the "reserve" proteins in the form of amides.

During the period of active growth, the water-soluble non-protein nitrogen, including the amino N, again increases. The fact that the amide N simultaneously decreases may indicate its connection with the utilization of amide N in protein synthesis. At times throughout the cycle the amide and amino N show inverse relationships. Since there is evidence (**34**) to indicate that in the germination of seeds amide N is formed from amino N, some such relationship may hold throughout in these experiments but may be masked at times by the difficulties involved in drawing sharp distinction between the groups. When active growth is over both the total water-soluble nitrogen and the non-protein nitrogen, including the amino and amide N, decline rapidly until the fall evacuation from the leaves commences. During this process of migration of nitrogen both the water-soluble and non-protein nitrogen increase up to the last sampling (November 20). The course of the fluctuation of the non-bearing spurs follows that of the one- and two-year branch growth.

During the so-called "dormant" period the amide N has increased but the amino N has decreased. A small amount (0.13 gm.) of a substance corresponding to the properties of asparagine was isolated from the 1923 dormant branch growth by a method to be given in a later paper.

Since asparagine contains both the $-CO \cdot NH_2$ and $-NH_2$ groups, the latter in the α-position, it is clear that other amino-acids are (as would be expected) present; otherwise the amide and amino N would always parallel one another throughout the cycle. In a later investigation an attempt will be made to identify the amines and amides present.

Summary

The quantitative changes of the principal nitrogen fractions in the non-bearing spurs and in the one- and two-year branch growths and leaves may be summarized:

I. PERIOD OF BUD SWELLING

(*a*) *Branches and non-bearing spurs.*—Total nitrogen, total water-soluble nitrogen, non-protein nitrogen, amino N and "rest" N increase; amide N decreases.

II. PERIOD OF COMMENCEMENT OF VEGETATIVE GROWTH

(*a*) *Branches and non-bearing spurs.*—Total nitrogen, total water-soluble nitrogen, non-protein nitrogen and amino N decrease; amide N increases.

III. PERIOD OF ACTIVE GROWTH

(*a*) *Branches and non-bearing spurs.*—Total nitrogen, total water-soluble nitrogen, and amino N increase; amide N decreases.

IV. PERIOD OF CESSATION OF ACTIVE GROWTH

(*a*) *Branches and spurs.*—Total nitrogen, total water-soluble nitrogen, non-protein nitrogen, amino N and "rest" N decrease; amide N increases.

(*b*) *Leaves.*—For the first period to the middle of August, the total nitrogen, total water-soluble nitrogen, non-protein nitrogen, amino N and amide N decreases; the "rest" N remains unchanged. During the second period to the middle of September, all the aforementioned fractions increase except the "rest" N which decreases.

V. PERIOD OF CHLOROPHYLL DEGENERATION

(*a*) *Branches and non-bearing spurs.*—Total nitrogen, total water-soluble nitrogen, non-protein nitrogen and amide N increase; amino N decreases.

(*b*) *Leaves.*—Total nitrogen, total water-soluble nitrogen, non-protein nitrogen and amino N decrease; amide N and "rest" N increase.

From these variations certain specific facts showing some of the more definite relationships have been discussed. These are:

1. The total nitrogen and the various partition fractions of the leaves vary with growth, decreasing when growth is very rapid. The total water-soluble nitrogen, the non-protein nitrogen and the amino N parallel the total nitrogen throughout the cycle; the amide N and the "rest" N tend to vary inversely as these fractions. Since the total nitrogen parallels the insoluble nitrogen and since the latter consists chiefly of the insoluble cytoplasmic protein (**33**), this may be regarded as supporting Chibnall's contention (**3**) that amino N is connected with protein synthesis and the "rest" N with protein degradation.

2. The total nitrogen and the partition groups of the "*wood*" *growth* (spurs and branches) also vary with growth, increasing when it is rapid.

The total nitrogen decreases rapidly from the period of bud swelling to the period of full bloom, and the amount of the decrease is indicative of the demands of the young shoots for nitrogen stored in the phloem.

3. The indications are that during bud swelling the reserve proteins are transported to the actively growing parts in the form of amino-acids.

4. There can be no question as to the phenomenon of autumnal migration of nitrogen from the leaves to the branches in this species. Storage is mainly in the one- and two-year growths.

5. In the aerial parts nitric N (nitrates) was found only at one period, *viz.*, in the buds just as they were opening.

6. In this species nitrates are transformed to amino-acids for the most part in the fine roots.

7. The soluble protein nitrogen is very small throughout the cycle.

8. Ammonia nitrogen is very low throughout the cycle.

9. Nitrogen equilibrium is just about maintained by the application of 5 lbs. of $NaNO_3$ to a 15-year-old Stayman Winesap tree growing in sod.

10. The importance of the possible rôle by the "rest" N compounds is pointed out. The results tend to confirm CHIBNALL's theory that they are connected with protein degradation.

11. Owing to the small quantities not only of soluble proteins but also of the total water-soluble nitrogenous products, this species is not suitable for investigations on the mechanism of protein synthesis.

The writer desires to thank Professors C. A. SHULL and H. W. POPP for suggestions and criticisms relating to the manuscript and to acknowledge the assistance of Mr. C. A. KERN in the determination of the imbibitional and hygroscopic water, and of MISS ETHEL GINGRICH in the computations of the analytical data.

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1927, xxiv, pp. 953-955

3654

Vitamin Studies XV. Assimilation of Vitamins A and D in Presence of Mineral Oil.*

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In our work on vitamin A it became necessary to dissolve known weights of butter fat and cod liver oil in vitamin-free oils, in order that the feeding of small amounts of vitamin-containing oils might be facilitated. We thought it would be of interest to observe the effect of mineral or paraffin oil on the assimilation of the fat-soluble vitamins, inasmuch as this type of oil should be a solvent for fat-soluble substances. Since the mineral oils are not absorbed in the gastro-intestinal tract, it is to be expected that the mineral oil should remove much of the oil-soluble materials with which it comes in contact.

In the vitamin A studies all rats were placed on the irradiated casein-dextrin ration[1] with daily additions of 0.4 grams of Fleischmann's special dried yeast. When the animals had depleted their storage of vitamin A, which occurred at about the 37th day of the preliminary feeding period, the various oil mixtures were fed separate from the ration in the daily dosages described above. All mineral oil-butter fat mixtures were made in the proportion, by weight, of mineral oil 2.5 to 1.0 of butter fat, 7 drops of the mixture containing .0666 grams of butter fat.

The first group of animals was fed a curative dose carrying .0666 grams of butter fat. None of these animals responded to the mixture, xerophthalmia developed and three of the animals died. At this point the mineral oil was removed and the butter fat feeding was continued. All animals began to grow and the ophthalmic condition disappeared. (See Curve I).

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* Published by permission of the Director of the Agricultural Experiment Station as Scientific Paper No. 430.

Three animals were given similar treatment except that the mineral oil-butter fat mixture was fed at a 14 drop level, which was equivalent to 4 drops of butter fat weighing .1333 grams. One animal died and the others failed to grow satisfactorily. At the ninth day the mineral oil was removed and the butter fat feeding was continued with an immediate response in growth. (See Curve II).

A third group was fed in the same manner, except that the daily allotment of the oil mixture was increased to 21 drops, which represented 6 drops or 0.2 grams of butter fat. These animals also failed to grow and the mineral oil was again removed and growth was resumed. (See Curve III.)

The fourth group was given the same treatment, using the oil mixture which contained daily additions of .1333 grams of butter fat. These animals were allowed to remain on experiment for 56 days, while the mineral oil was alternately added and removed at stated intervals. The growth curves of the animals rose and fell with the omission and addition of mineral oil. (See Curve IV). To conserve space only representative curves are given.

It is quite evident from the results obtained that mineral oils possess the property of dissolving vitamin A from food materials, thereby depriving the body of this vitamin, even though vitamin A be present in excess of the body needs.

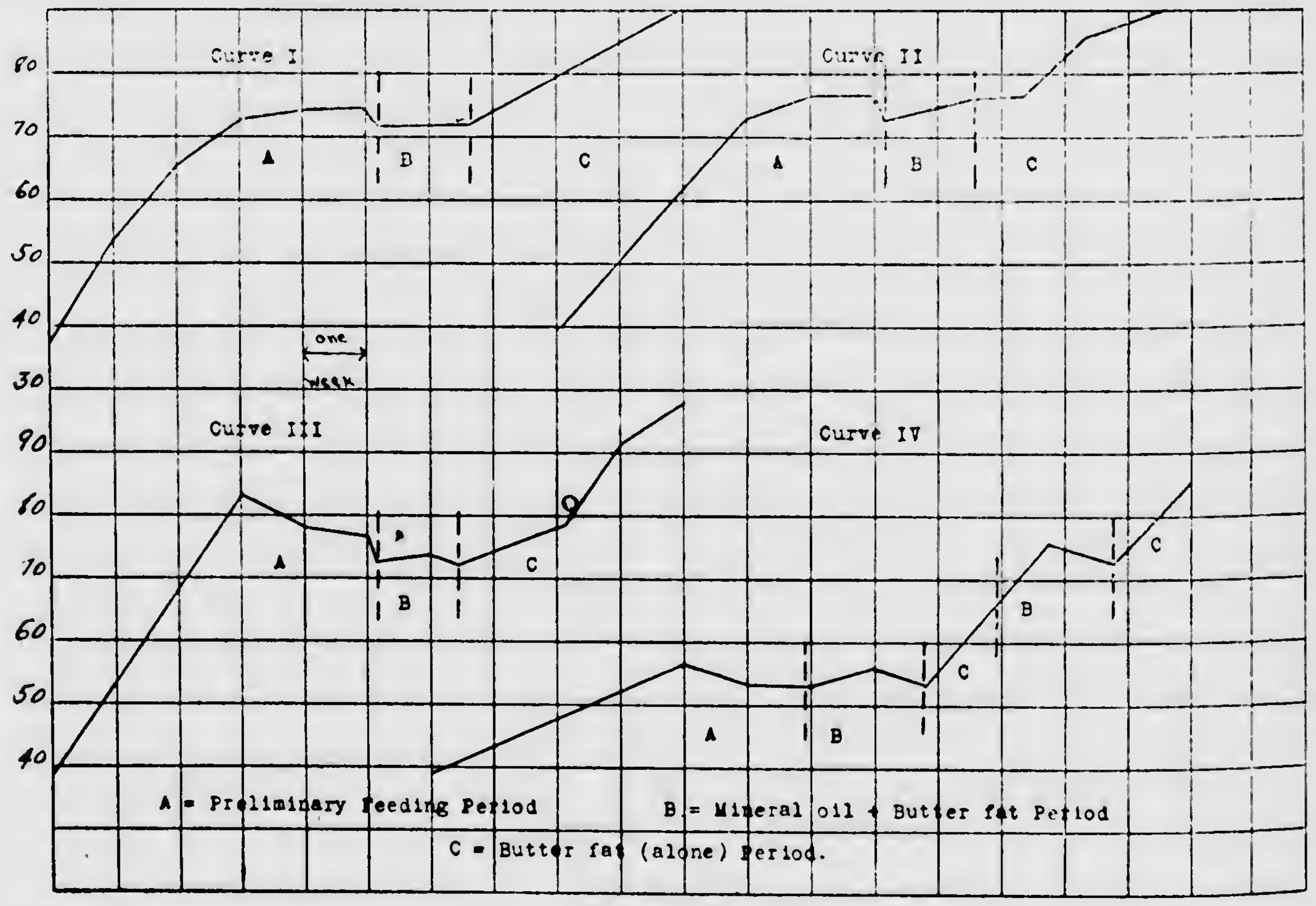

CHART 1.

The fifth group, consisting of 10 rats, was placed on experiment to study the calcifying potency of cod liver oil when fed in the presence of mineral oil. The same technique was followed as described in a previous paper[2] using the Steenbock yellow corn ration.

A mineral oil-cod liver oil mixture was prepared of 3.56 parts (by weight) of mineral oil to 1 part of cod liver oil. Previous experiments with this sample of cod liver oil (4 drops daily) had yielded 41.5 per cent of (femur) bone ash after the animals had subsisted on the Steenbock ration for 21 days. In the study of the effect of mineral oil the animals received daily dosages of 15 drops of the mixture which contained 4 drops of cod liver oil weighing .1180 grams.

When the rats had subsisted for 21 days on the Steenbock ration, and the daily allotment of 15 drops of oil mixture, all animals were chloroformed and the femur bones were dried, extracted with alcohol and ether and ashed. The average percentage of ash obtained was 40.80, which approximates the results we obtain when cod liver oil is fed alone. Line tests were performed on all of the animals and the pictures indicated a degree of calcification identical with that obtained when cod liver oil is fed in the absence of mineral oil, thereby substantiating the results obtained by the ash method.

It is clear that mineral oil may act as a solvent for vitamin A, thereby depleting the ingested foods of their supply of this vitamin, but we have obtained no evidence to show that the calcifying potency of cod liver oil is appreciably affected by the presence of mineral oil. It is entirely possible that increased amounts of mineral oil might show a solvent effect on the antirachitic factor.

Work is now in progress to study the entire matter in greater detail, and with particular reference to the influence of mineral oil upon the digestion of fats and oils. We are also planning to study the effect of diets rich in vitamin deficient fats with the view of determining whether or not undigested glycerides may not have a solvent action on the fat-soluble vitamins in the food and body tissues.

Note: After this paper had been prepared and the work announced at the Rochester meeting of the Federated Societies for Experimental Biology (April 11-16, 1927), the work of Burrows, M. T., and Farr, W. K., appeared in this PROCEEDINGS (xxiv, 719, April, 1927), substantiating the findings described in this paper.

This is a preliminary report.

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[1] Dutcher, R. A., and Kruger, J. H., *J. Biol. Chem.*, 1926, lxix, 277.

[2] Dutcher, R. A., Creighton, M., and Rothrock, H., *J. Biol. Chem.*, 1925, lxvi, 401.

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NITROGENOUS METABOLISM OF *PYRUS MALUS* L.

IV. THE EFFECT OF SODIUM NITRATE APPLICATIONS ON THE TOTAL NITROGEN AND ITS PARTITION PRODUCTS IN THE LEAVES, NEW AND ONE YEAR BRANCH GROWTH THROUGHOUT A YEAR'S CYCLE[1]

WALTER THOMAS

(WITH SEVEN FIGURES)

Introduction

The third paper of this series (7) gave the results of an investigation on the quantitative changes occurring in the various nitrogen fractions throughout a year's cycle in a Stayman Winesap tree, 15 years old, in the "off" year, growing in sod in the College Experimental Orchard.

Although no evidence to support the claim of certain investigators that amino-acids act as catalyzers in accelerating the rate of growth was obtained, nevertheless the results showed that amino-acids play a specific rôle. They appear to be the form in which nitrogen is carried from the roots to the metabolically active parts and, with the amides, are connected with the synthesis and utilization of proteins. Moreover, it was also shown that the unclassified or "rest" N compounds, concerning the nature of which little is known, may play a rôle as important as that of amino-acids, amines and amides, because of their apparent function in protein degradation. Finally, evidence has been produced (8) showing that the reduction of nitrates and the formation of amino-acids take place in this species for the most part in the fine roots; hence, a knowledge of the internal effects produced by nitrogenous fertilizers can only be obtained by following the fluctuation of the products or substances into which the NO_3 ion is transformed.

That a knowledge of the nitrogen distribution in the woody perennials is highly desirable is recognized by several investigators. Thus, PROEBSTING (4) has suggested that not the total nitrogen but some fraction of it might have to be considered in all attempts to correlate the relation of nutrients to the activity of the cambium. POTTER and KRAYBILL (3), discussing the behavior of bearing as compared to non-bearing spurs of apple trees with respect to the correlation between composition and spur performance, state that the determination of the different forms of nitrogen is "a difficult or impossible task" but add "that if progress can be made in differentiating the forms of nitrogen available to spurs, it will be of greatest value in interpreting results." The present pioneer investigations have shown that the task is not impossible; it is, however, difficult, laborious, and time-consuming and will of necessity require a large force of workers in any attempt to correlate the performance of a large number of trees or plants receiving different fertilizer treatments with the forms of nitrogen available to the more metabolically active parts.

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[1] Published with the approval of the Director of the Agricultural Experiment Station as scientific paper no. 432.

It is uncertain, with our present limited knowledge, to what extent the determination of the nitrogen distribution in plants can be applied to practical agriculture. Thus far, an insight has been obtained only into the quantitative changes taking place during a year's cycle of the water-soluble nitrogen fractions. The possibilities and limitations of nitrogen distribution investigations in this species are clearly defined, and more especially will they help to clarify all speculations relative to the utilization of any of the nitrogen fractions in the development of the carbohydrate-nitrogen relations in investigations relating to vegetative growth and flower formation.

Digressing for the moment, it may be pointed out here that the logical point of attacking such problems as, for example, the factors influencing fruit bud formation, which so many plant physiologists and horticulturists are at present attempting to solve, may be to ascertain first of all the internal conditions that cause differences in vigor, this being the external expression of the internal factors affecting the rate of metabolism, which, in turn, is limited by the rate of absorption of nutrients by the root system. The present partition results offer suggestions in this respect.

In the earlier metabolism investigations of the writer (**6**) the nitrogen partition work was carried out on one tree in the absence of the disturbing factor of developing fruit. This investigation was extended in 1924–25 to include a comparison of the course of the various nitrogen fractions throughout a year's cycle in two trees of the same variety and age growing along side one another in a homogenous soil, one of which received sodium nitrate applications and the other none. The question may be asked, what basis is there to justify a comparison of analytical results in which one tree and one tree only is compared with another of the same age and variety growing beside it in a homogenous soil? In other words, can it be postulated that the performance of the tree receiving $NaNO_3$ additions would have paralleled the untreated tree with which it is compared had the former received no such additions; or, if both trees had received the same nitrogen additions would the responses indicated by the analytical data be identical? From the results of investigations carried out by the writer in 1918–20, there appears to be little doubt that if the sampling is carried out on the

principles already stated (**7**) such comparisons as are here attempted are scientifically sound. The differences in the total nitrogen and its partition products observed in the present investigation, therefore, can logically be attributed to the addition of sodium nitrate to the one tree and not to the other.

Materials and methods

Two Stayman Winesap trees, 16 years old, growing adjacent to one another in the College Orchard, both of which had always been subjected to the same general treatment, and which resulted in very similar growth and reproductive responses, were used in this investigation. One of these trees, no. E–20, was treated with 10 pounds of sodium nitrate on April 20, 1924, just after the buds were commencing to swell, and again with another 10 pounds on June 8, 1924, at the initiation of fruit bud formation. This quantity is four times the application normally given, the object being to accentuate whatever internal differences might be produced in the nitrogen metabolism of the trees by the nitrate additions as indicated by the partition results. The external differences that existed were shown by the much darker color of the foliage and greater vegetative extension of the treated tree. Reproduction, as indicated by the yields, was 8 per cent. less on the treated tree, which would lead to the conclusion that if the theory of carbohydrate-nitrogen ratios holds, the excessive nitrate applications have tended to shift the treated trees from class III to class II in Kraus and Kraybill's classification.

The collection of samples was carried out under favorable meteorological conditions in the early morning, in the manner already described (**7**). The collection was made on ten different dates between April 4, 1924, and November 11, 1924, the samples being taken to the laboratory immediately and dried in the manner already indicated (**6**).

As in the earlier experiments, no correlation could be found between the total nitrogen or any of its fractions and any of the climatic factors. However, a summary of the weather conditions is appended as a matter of record.

The wood and bark separation problem.—In all collections dating from June 11, 1924, the wood and bark of the branch growths were separated before preserving the samples by desiccation, but, as pointed out in the third paper (**7**), it was found that no special advantage is to be derived from this procedure and that, moreover, the interpretation of the nitrogen partition results is not affected by such separations. Also, owing to the relatively small quantity of each type of branch tissues available for the partition work, it was found in many cases that there was either insufficient "wood" or "bark" to carry out satisfactorily the analytical work upon them separately. Most of the work, therefore, was carried out on samples of

TABLE I

SUMMARY OF METEOROLOGICAL DATA

DATES	MEAN MAXIMUM	MEAN MINIMUM	RAINFALL	SUNSHINE	HUMIDITY
1924	Deg. F.	Deg. F.	Inches	Per cent.	Per cent.
April 4–April 21	53.5	34.8	3.26	59.4	68.7
April 22–April 28	62.3	36.7	0.28	83.3	63.9
April 29–May 13	62.5	43.5	4.65	54.0	79.2
May 14–May 22	59.9	43.0	1.33	41.9	76.5
May 23–June 11	63.2	45.9	2.35	48.9	77.0
June 12–July 16	76.7	56.0	7.07	64.7	79.3
July 17–August 28	78.4	56.5	4.30	76.3	76.3
August 29–October 7	68.5	46.9	4.54	42.9	79.8
October 8–November 11	60.5	37.2	0.16	....	69.6

"wood" and "bark" combined in the proportions in which they were present in the original sample. There is, therefore, as far as the principal nitrogen fractions are concerned, little danger of a deficiency due to localization of the metabolically active nitrogen fractions in some tissues at the expense of others. Consequently, all analytical data have been re-calculated to the original basis as if wood and bark had been ground up together.

THE METHOD IN DETAIL.—The extractions with water were carried out in the manner outlined in the first paper (**6**).

The two fractionation schemes adopted in these metabolism investigations have already been discussed in detail (**7**). The second fractionation scheme was adopted in the present work, as fewer separations are involved, a weighty consideration where a large number of samples is to be examined, in spite of the fact that a consideration of the accuracy and limits of error as given in table III indicates that this second scheme is subject to greater analytical errors than the first scheme. However, for the present comparative studies the second scheme serves quite well.

In the non-protein filtrates from the colloidal ferric hydroxide precipitation (**6**), the following fractions were determined:—Ammonia N, amide N (asparagine and possibly glutamine N), basic N, a-mono-amino N, and also humin and melanin N from the amide and basic nitrogen determinations. The sum of these nitrogen fractions subtracted from the non-protein nitrogen gives the "rest" nitrogen.

1. *Hygroscopic water.*—This was determined on a two gram sample dried at 110° C. for 24 hours.

2. *Total water-soluble N.*—This fraction was determined in the usual way by the Kjeldahl method on 200 cc. aliquots, adopting standard methods and precautions for estimating small quantities of nitrogen.

3. *Ammonia N.*—The ammonia N was determined on a 500 cc. aliquot before treatment with colloidal iron and for reasons already given (**7**).

4. *Non-protein N.*—This was determined in the same manner as the total water-soluble nitrogen on 200 cc. aliquots of the filtrates from the colloidal ferric hydroxide precipitation.

5. *Amide N (asparagine and possibly glutamine N).*—Up to this stage the fractionation method is similar to that adopted in the earlier investigations (**7**); the point of departure is made at this stage. The determination of amide N is carried out essentially by SACHSSE's method (**5**), *i.e.*, by adding a definite quantity of HCl (sufficient to give a 4 per cent. solution) to the whole of the non-protein N filtrate remaining after the removal of the aliquots for the determination of nitrogen (which usually amounts to 1,600 cc.), and then hydrolyzing for three hours. The solution remaining after the removal of the HCl *in vacuo* is diluted to about 300 cc. with ammonia-free distilled water and distilled *in vacuo* with solid CaO (**7**). The asparagine N (with possibly some glutamine N) is obtained by subtracting the free ammonia, determined as previously indicated, from the amount obtained in this determination, *i.e.*, after hydrolysis. Proteoses were present in too small a quantity to affect the amide N results.

6. *Humin N and $Ca(OH)_2$ melanin N and basic N.*—The solution remaining in the flask from the amide N determination is filtered from the humin and $Ca(OH)_2$ melanin N, washed with hot water until free from chlorides, and the nitrogen content of these precipitates determined.

7. *The basic N.*—HAUSMANN's method (**1**) with modifications (**2, 9**) as indicated later was adopted. The filtrate and washings were concentrated *in vacuo* to about 80 cc. and then transferred to a beaker, the contents being cooled to 20° C. Afterwards 2.5 cc. concentrated H_2SO_4 were slowly added, followed by a solution containing 20 gm. of phosphotungstic acid and 5 gm. concentrated H_2SO_4 per 100 cc., the mixture being added drop by drop. Six cc. of this phosphotungstic acid mixture were quite sufficient in the present work. Care is to be taken to avoid more than a slight excess of the phosphotungstic acid solution; otherwise difficulty will be encountered in the subsequent amino-acid determination, owing to precipitation of some of the phosphotungstic acid. The solutions were diluted to 200 cc., heated on the water bath until the precipitates of the bases were dissolved, and then allowed to stand for two or three days. The granular precipitate was filtered off and washed with a small quantity of phosphotungstic acid solution containing 2.5 gm. phosphotungstic acid and 5 gm. concentrated H_2SO_4 per 100 cc. The technique of washing these precipitates was carried out in accordance with OSBORNE and HARRIS's (**2**) recommendations. VAN SLYKE (**9**) gives additional observations on the care in precipitating and washing

TABLE II

The percentages of imbibitional and total water

One year branch growth (tree no. E–20)

Series no.	Collecting date	Description	Fresh weight	Moisture free weight	Imbibitional water	Total water
			gm.	gm.	Per cent.	Per cent.
76	April 4, 1924	(wood and bark)	70.0	35.7	49.0	58.1
77	April 21, 1924	(wood and bark)	121.0	58.1	52.0	59.0
92	April 28, 1924	(wood and bark)	55.0	27.0	50.9	56.9
114	May 13, 1924	(wood and bark)	73.0	35.0	52.0	58.4
142	May 22, 1924	(wood and bark)	51.0	22.0	56.9	57.8
161	June 11, 1924	(wood)	55.5	28.8	48.1	51.7
162	June 11, 1924	(bark)	43.8	17.9	59.1	60.7
185A	July 16, 1924	(wood)	78.8	43.7	44.4	46.8
185B	July 16, 1924	(bark)	56.6	22.6	58.8	61.8
196	August 28, 1924	(wood)	70.1	39.5	43.7	46.9
196A	August 28, 1924	(bark)	52.6	22.6	57.0	60.9
203A	October 7, 1924	(wood)	88.3	55.6	37.0	45.0
203B	October 7, 1924	(bark)	63.1	28.2	55.3	57.3
237	November 11, 1924	(wood)	123.0	71.1	42.2	44.7
238	November 11, 1924	(bark)	78.4	38.4	50.8	54.0

One year branch growth (tree no. E–22)

Series no.	Collecting date	Description	Fresh weight	Moisture free weight	Imbibitional water	Total water
			gm.	gm.	Per cent.	Per cent.
60	April 4, 1924	(wood and bark)	69.0	35.0	49.3	53.0
68	April 21, 1924	(wood and bark)	118.0	56.0	52.5	56.2
86A	April 28, 1924	(wood and bark)	69.0	34.0	50.7	60.0
105	May 13, 1924	(wood and bark)	81.0	37.0	54.3	60.4
124	May 22, 1924	(wood and bark)	53.3	21.6	59.4	60.0
154	June 11, 1924	(wood)	52.5	33.0	37.7	42.9
155	June 11, 1924	(bark)	54.0	24.3	55.0	60.2
180A	July 16, 1924	(wood)	72.4	41.0	43.6	47.8
180B	July 16, 1924	(bark)	51.8	21.4	56.4	60.9
199A	August 28, 1924	(wood)	71.2	40.4	43.2	44.2
199B	August 28, 1924	(bark)	53.6	26.7	47.5	58.7
215A	October 7, 1924	(wood)	90.6	54.4	40.0	43.1
215B	October 7, 1924	(bark)	65.0	36.5	56.2	57.4
218	November 11, 1924	(wood)	120.0	42.4	35.4	40.6
217	November 11, 1924	(bark)	75.0	33.7	52.2	57.0

TABLE II (*Continued*)

The percentages of imbibitional and total water

New growth (tree no. E–20)

Series no.	Collecting date	Description	Fresh weight	Moisture free weight	Imbibitional water	Total water
			gm.	gm.	Per cent.	Per cent.
160	June 11, 1924		50.0	14.4	66.2	71.2
185	July 16, 1924	(wood)	32.0	12.0	62.5	65.4
186	July 16, 1924	(bark)	46.0	16.0	65.2	68.1
191	August 28, 1924	(wood)	30.0	16.0	46.6	50.2
192	August 28, 1924	(bark)	31.0	14.0	54.8	57.9
206	October 7, 1924	(wood)	52.3	31.0	40.7	44.1
207	October 7, 1924	(bark)	66.6	21.5	67.7	69.8
217	November 11, 1924	(wood)	68.0	42.5	37.5	43.9
218	November 11, 1924	(bark)	51.0	31.2	38.8	44.5

New growth (tree no. E–22)

Series no.	Collecting date	Description	Fresh weight	Moisture free weight	Imbibitional water	Water Total
			gm.	gm.	Per cent.	Per cent.
153	June 11, 1924		64.0	20.8	67.5	72.9
188A	July 16, 1924	(wood)	31.6	12.3	60.8	64.2
189	July 16, 1924	(bark)	46.7	17.7	61.8	65.6
196A	August 28, 1924	(wood)	37.0	21.0	43.2	47.3
196	August 28, 1924	(bark)	40.0	19.0	52.5	54.2
212	October 7, 1924	(wood)	46.9	29.0	38.0	42.0
211	October 7, 1924	(bark)	40.5	21.1	48.1	51.6
230	November 11, 1924	(wood)	62.6	38.0	37.7	41.9
231	November 11, 1924	(bark)	54.2	27.6	49.1	52.6

these phosphotungstic acid precipitates. However, no special difficulty was encountered in washing them. Since the precipitates were small, care had to be exercised to employ small filters and to avoid more than two or three washings, using suction and a wash solution cooled to 0° C. In this respect the technique differs from that employed in protein work, in which the quantities of basic nitrogen obtained from 2 to 3 gm. of protein are comparatively large.

Nitrogen was determined in these precipitates in the usual way, the precipitate and filter being transferred to the Kjeldahl flash direct, since it was not necessary to decompose the basic phosphotungstic precipitates, inas-

TABLE II (*Concluded*)

THE PERCENTAGES OF IMBIBITIONAL AND TOTAL WATER

LEAVES (TREE NO. E–20)

SERIES NO.	COLLECTING DATE	FRESH WEIGHT	MOISTURE FREE WEIGHT	IMBIBITIONAL WATER	TOTAL WATER
		gm.	gm.	Per cent.	Per cent.
138	May 13, 1924	50.0	13.0	74.0	77.0
140	May 22, 1924	70.3	20.0	72.6	76.4
166	June 11, 1924	97.0	32.0	67.0	70.0
189	July 16, 1924	204.0	82.0	59.8	62.6
195	August 28, 1924	144.0	60.0	58.3	60.7
210	October 7, 1924	170.0	77.0	54.7	57.9
222A	November 11, 1924	40.0	19.3	51.8	56.3

LEAVES (TREE NO. E–22)

SERIES NO.	COLLECTING DATE	FRESH WEIGHT	MOISTURE FREE WEIGHT	IMBIBITIONAL WATER	TOTAL WATER
		gm.	gm.	Per cent.	Per cent.
106	May 13, 1924	49.0	16.0	66.7	72.3
129	May 22, 1924	73.3	18.0	75.4	77.0
153A	June 11, 1924	85.0	24.3	60.4	65.6
180	July 16, 1924	203.0	82.0	59.6	65.6
199	August 28, 1924	214.0	89.0	58.4	61.4
215	October 7, 1924	168.0	63.5	62.2	65.2
233A	November 11, 1924	38.0	15.5	59.2	64.3

much as the determination of the diamino acids—histidine, arginine and lysine—was not under consideration in this investigation.

8. *a-Mono-amino N.*—The filtrate and washings from the phosphotungstic acid precipitation were made alkaline (pH 7.5) with 50 per cent. NaOH and then acid (pH 6.0) with acetic acid, concentrated *in vacuo* to about 80 cc., *i.e.*, to the point at which salts begin to separate out, and finally made up to a definite volume (usually 100 cc.). The a-mono-amino N was determined in the usual way in the VAN SLYKE micro-apparatus.

9. *"Rest" N.*—This was calculated by difference, as already described.

Experimental results

A description of the samples with their fresh and moisture free weights is given in table II, and the partition results in table III.

TABLE III

TOTAL NITROGEN AND TOTAL WATER-SOLUBLE NITROGEN IN THE NEW (1924) BRANCH GROWTH, THE ONE YEAR (1923) BRANCH GROWTH AND LEAVES, TOGETHER WITH THE DISTRIBUTION OF THE NON-PROTEIN CONSTITUENTS EXPRESSED AS PERCENTAGES OF FRESH AND MOISTURE FREE WEIGHTS, RESPECTIVELY

ONE YEAR BRANCH GROWTH (TREE NO. E–20)

SERIES NO.	COLLECTING DATE	TOTAL N	TOTAL WATER-SOLUBLE N	TOTAL NON-PROTEIN N	AMMONIA N	AMINO N	AMIDE N	BASIC N	HUMIN N	RESIDUAL N
			As percentages of fresh weight of material							
76	April 4, 1924	0.263	0.0370	0.0317	0.0059	0.0041	0.0029	0.0046	0.0015	0.0126
77	April 21, 1924	0.308	0.0463	0.0380	0.0005	0.0070	0.0094	0.0057	0.0028	0.0197
92	April 28, 1924	0.329	0.0698	0.0541	0.0022	0.0084	0.0086	0.0108	0.0028	0.0216
114	May 13, 1924	0.225	0.0582	0.0458	0.0012	0.0064	0.0042	0.0083	0.0027	0.0229
142	May 22, 1924	0.287	0.0789	0.0429	0.0019	0.0120	0.0080	0.0096	0.0029	0.0084
161 162	June 11, 1924	0.248	0.0443	0.0349	0.0013	0.0035	0.0049	0.0066	0.0026	0.0159
185A 185B	July 16, 1924	0.310	0.0733	0.0455	0.0014	0.0113	0.0127	0.0042	0.0035	0.0122
196	August 28, 1924	0.274	0.0643	0.0531	0.0020	0.0070	0.0110	0.0107	0.0021	0.0201
203A 203B	October 7, 1924	0.298	0.0697	0.0598	0.0017	0.0080	0.0134	0.0099	0.0027	0.0239
237 238	November 11, 1924	0.346	0.0852	0.0716	0.0020	0.0130	0.0140	0.0110	0.0030	0.0275
			As percentages of moisture free weight of material							
76	April 4, 1924	0.628	0.0835	0.0756	0.0140	0.0098	0.0070	0.0110	0.0035	0.0303
77	April 21, 1924	0.750	0.1130	0.0929	0.0012	0.0170	0.0230	0.0140	0.0068	0.0481
92	April 28, 1924	0.764	0.1620	0.1256	0.0050	0.0195	0.0200	0.0250	0.0066	0.0500
114	May 13, 1924	0.540	0.1400	0.1101	0.0030	0.0155	0.0100	0.0200	0.0066	0.0550
142	May 22, 1924	0.680	0.1870	0.1017	0.0045	0.0285	0.0190	0.0228	0.0069	0.0200
161 162	June 11, 1924	0.560	0.1000	0.0788	0.0030	0.0080	0.0110	0.0150	0.0058	0.0360
185A 185B	July 16, 1924	0.660	0.1560	0.0968	0.0030	0.0240	0.0270	0.0090	0.0075	0.0260
196	August 28, 1924	0.545	0.1280	0.1057	0.0040	0.0140	0.0220	0.0214	0.0042	0.0400
203A 203B	October 7, 1924	0.600	0.1400	0.1200	0.0035	0.0160	0.0270	0.0200	0.0055	0.0480
237 238	November 11, 1924	0.690	0.1700	0.1430	0.0040	0.0260	0.0280	0.0220	0.0060	0.0550

TABLE III (*Continued*)

TOTAL NITROGEN AND TOTAL WATER-SOLUBLE NITROGEN IN THE NEW (1924) BRANCH GROWTH, THE ONE YEAR (1923) BRANCH GROWTH AND LEAVES, TOGETHER WITH THE DISTRIBUTION OF THE NON-PROTEIN CONSTITUENTS EXPRESSED AS PERCENTAGES OF FRESH AND MOISTURE FREE WEIGHTS, RESPECTIVELY

ONE YEAR BRANCH GROWTH (TREE NO. E–22)

SERIES NO.	COLLECTING DATE	TOTAL N	TOTAL WATER-SOLUBLE N	TOTAL NON-PROTEIN N	AMMONIA N	AMINO N	AMIDE N	BASIC N	HUMIN N	RESIDUAL N
			As percentages of fresh weight of material							
60	April 4, 1924	0.299	0.0384	0.0286	0.0066	0.0045	0.0034	0.0047	0.0010	0.0086
68	April 21, 1924	0.279	0.0520	0.0389	0.0026	0.0068	0.0057	0.0064	0.0013	0.0160
86A	April 28, 1924	0.288	0.0560	0.0395	0.0022	0.0076	0.0047	0.0058	0.0032	0.0160
105	May 13, 1924	0.166	0.0416	0.0309	0.0016	0.0031	0.0014	0.0041	0.0030	0.0168
124	May 22, 1924	0.186	0.0385	0.0375	....	....	....	....	....	....
154 155	June 11, 1924	0.171	0.0353	0.0290	0.0015	0.0013	0.0027	0.0030	0.0036	0.0169
180A 180B	July 16, 1924	0.153	0.0387	0.0257	0.0016	0.0025	0.0025	0.0027	0.0036	0.0128
199A 199B	August 28, 1924	0.158	0.0321	0.0211	0.0017	0.0015	0.0048	0.0032	0.0025	0.0075
215A 215B	October 7, 1924	0.151	0.0357	0.0262	0.0014	0.0028	0.0061	0.0034	0.0029	0.0096
228 229	November 11, 1924	0.208	0.0432	0.0352	0.0019	0.0045	0.0071	0.0040	0.0024	0.0152
			As percentages of moisture free weight of material							
60	April 4, 1924	0.635	0.0818	0.0608	0.0140	0.0095	0.0072	0.0100	0.0021	0.0182
68	April 21, 1924	0.638	0.1187	0.0887	0.0060	0.0155	0.0130	0.0147	0.0030	0.0365
86A	April 28, 1924	0.720	0.1400	0.0988	0.0055	0.0190	0.0118	0.0145	0.0080	0.0400
105	May 13, 1924	0.480	0.1203	0.0893	0.0046	0.0090	0.0040	0.0118	0.0088	0.0485
124	May 22, 1924	0.465	0.1051	0.0865	0.0040	0.0120	0.0085	0.0165	0.0080	0.0400
154 155	June 11, 1924	0.405	0.0832	0.0687	0.0035	0.0030	0.0065	0.0072	0.0085	0.0400
180A 180B	July 16, 1924	0.340	0.0860	0.0570	0.0035	0.0055	0.0055	0.0060	0.0080	0.0285
199A 199B	August 28, 1924	0.315	0.0640	0.0422	0.0034	0.0030	0.0095	0.0063	0.0050	0.0150
215A 215B	October 7, 1924	0.314	0.0740	0.0543	0.0030	0.0058	0.0126	0.0070	0.0060	0.0200
228 229	November 11, 1924	0.437	0.0910	0.0740	0.0040	0.0095	0.0150	0.0085	0.0050	0.0320

TABLE III (*Continued*)

TOTAL NITROGEN AND TOTAL WATER-SOLUBLE NITROGEN IN THE NEW (1924) BRANCH GROWTH, THE ONE YEAR (1923) BRANCH GROWTH AND LEAVES, TOGETHER WITH THE DISTRIBUTION OF THE NON-PROTEIN CONSTITUENTS EXPRESSED AS PERCENTAGES OF FRESH AND MOISTURE FREE WEIGHTS, RESPECTIVELY

NEW GROWTH (TREE NO. E–20)

SERIES NO.	COLLECTING DATE	TOTAL N	TOTAL WATER-SOLUBLE N	TOTAL NON-PROTEIN N	AMMONIA N		AMIDE N	BASIC N	HUMIN N	RESIDUAL N
			As percentages of fresh weight of material							
160	June 11, 1924	0.432	0.0490	0.0369	0.0013	0.0124	0.0078	0.0046	0.0013	0.0092
185 186	July 16, 1924	0.363	0.0818	0.0714	0.0035	0.0250	0.0089	0.0073	0.0035	0.0228
191 192	August 28, 1924	0.378	0.0771	0.0716	0.0010	0.0207	0.0128	0.0096	0.0034	0.0246
206 207	October 7, 1924	0.424	0.0979	0.0810	0.0031	0.0265	0.0148	0.0069	0.0032	0.0278
217 218	November 11, 1924	0.517	0.1114	0.0964	0.0023	0.0281	0.0201	0.0046	0.0040	0.0373
			As percentages of moisture free weight of material							
160	June 11, 1924	1.503	0.1700	0.1290	0.0045	0.0430	0.0270	0.0160	0.0045	0.0320
185 186	July 16, 1924	0.843	0.1900	0.1650	0.0082	0.0580	0.0206	0.0170	0.0082	0.0530
191 192	August 28, 1924	0.784	0.1600	0.1485	0.0020	0.0430	0.0265	0.0200	0.0070	0.0510
206 207	October 7, 1924	0.802	0.1850	0.1533	0.0058	0.0500	0.0280	0.0130	0.0060	0.0525
217 218	November 11, 1924	0.900	0.1940	0.1680	0.0040	0.0490	0.0350	0.0080	0.0070	0.0650

TABLE III (*Continued*)

TOTAL NITROGEN AND TOTAL WATER-SOLUBLE NITROGEN IN THE NEW (1924) BRANCH GROWTH, THE ONE YEAR (1923) BRANCH GROWTH AND LEAVES, TOGETHER WITH THE DISTRIBUTION OF THE NON-PROTEIN CONSTITUENTS EXPRESSED AS PERCENTAGES OF FRESH AND MOISTURE FREE WEIGHTS, RESPECTIVELY

NEW GROWTH (TREE NO. E–22)

SERIES NO.	COLLECTING DATE	TOTAL N	TOTAL WATER-SOLUBLE N	TOTAL NON-PROTEIN N	AMMONIA N	AMINO N	AMIDE N	BASIC N	HUMIN N	RESIDUAL N
			As percentages of fresh weight of material							
153	June 11, 1924	0.379	0.0278	0.0215	0.0012	0.0063	0.0033	0.0028	0.0023	0.0057
181										
182	July 16, 1924	0.199	0.0378	0.0309	0.0013	0.0079	0.0031	0.0046	0.0015	0.0125
196A										
196B	August 28, 1924	0.227	0.0414	0.0330	0.0019	0.0062	0.0041	0.0036	0.0027	0.0145
211										
212	October 7, 1924	0.276	0.0500	0.0377	0.0017	0.0062	0.0070	0.0037	0.0040	0.0193
230										
231	November 11, 1924	0.300	0.0527	0.0462	0.0013	0.0073	0.0087	0.0031	0.0029	0.0213
			As percentages of moisture free weight of material							
153	June 11, 1924	1.400	0.1026	0.0703	0.0043	0.0230	0.0120	0.0102	0.0086	0.0212
181										
182	July 16, 1924	0.605	0.1150	0.0940	0.0040	0.0240	0.0095	0.0140	0.0045	0.0380
196A										
196B	August 28, 1924	0.547	0.1000	0.0798	0.0045	0.0150	0.0100	0.0088	0.0065	0.0350
211										
212	October 7, 1924	0.557	0.1010	0.0762	0.0035	0.0125	0.0142	0.0075	0.0080	0.0390
230										
231	November 11, 1924	0.603	0.1089	0.0954	0.0030	0.0150	0.0180	0.0064	0.0060	0.0440

TABLE III (*Continued*)

TOTAL NITROGEN AND TOTAL WATER-SOLUBLE NITROGEN IN THE NEW (1924) BRANCH GROWTH, THE ONE YEAR (1923) BRANCH GROWTH AND LEAVES, TOGETHER WITH THE DISTRIBUTION OF THE NON-PROTEIN CONSTITUENTS EXPRESSED AS PERCENTAGES OF FRESH AND MOISTURE FREE WEIGHTS, RESPECTIVELY

LEAVES (TREE NO. E–22)

SERIES NO.	COLLECTING DATE	TOTAL N	TOTAL WATER-SOLUBLE N	TOTAL NON-PROTEIN N	AMMONIA N	AMINO N	AMIDE N	BASIC N	HUMIN N	RESIDUAL N
			As percentages of fresh weight of material							
138	May 13, 1924	0.830	0.0690	0.0575	0.0019	0.0092	0.0028	0.0120	0.0081	0.0219
140	May 22, 1924	0.806	0.0802	0.0658	0.0015	0.0145	0.0042	0.0165	0.0078	0.0217
166	June 11, 1924	0.774	0.0732	0.0660	0.0012	0.0138	0.0027	0.0165	0.0063	0.0219
189	July 16, 1924	0.804	0.1058	0.0954	0.0022	0.0204	0.0026	0.0224	0.0120	0.0370
195	August 28, 1924	0.817	0.1014	0.0880	0.0016	0.0137	0.0076	0.0157	0.0110	0.0385
210	October 7, 1924	0.777	0.0973	0.0869	None	0.0147	0.0084	0.0177	0.0164	0.0379
222A	November 11, 1924	0.677	0.0961	0.0861	None	0.0125	0.0118	0.0122	0.0059	0.0433
			As percentages of moisture free weight of material							
138	May 13, 1924	3.610	0.3000	0.2500	0.0085	0.0402	0.0121	0.0520	0.0350	0.0950
140	May 22, 1924	3.415	0.3400	0.2790	0.0065	0.0615	0.0180	0.0700	0.0330	0.0920
166	June 11, 1924	2.210	0.2440	0.2200	0.0040	0.0465	0.0092	0.0550	0.0310	0.0730
189	July 16, 1924	2.150	0.2830	0.2550	0.0058	0.0545	0.0070	0.0600	0.0320	0.0990
195	August 28, 1924	2.080	0.2580	0.2240	0.0040	0.0350	0.0194	0.0400	0.0280	0.0980
210	October 7, 1924	1.845	0.2310	0.2060	None	0.0350	0.0200	0.0420	0.0390	0.0900
222A	November 11, 1924	1.550	0.2200	0.1970	None	0.0285	0.0270	0.0280	0.0135	0.0990

TABLE III (*Concluded*)

TOTAL NITROGEN AND TOTAL WATER-SOLUBLE NITROGEN IN THE NEW (1924) BRANCH GROWTH, THE ONE YEAR (1923) BRANCH GROWTH AND LEAVES, TOGETHER WITH THE DISTRIBUTION OF THE NON-PROTEIN CONSTITUENTS EXPRESSED AS PERCENTAGES OF FRESH AND MOISTURE FREE WEIGHTS, RESPECTIVELY

LEAVES (TREE NO. E–20)

SERIES NO.	COLLECTING DATE	TOTAL N	TOTAL WATER-SOLUBLE N	TOTAL NON-PROTEIN N	AMMONIA N	AMINO N	AMIDE N	BASIC N	HUMIN N	RESIDUAL N
			As percentages of fresh weight of material							
106	May 13, 1924	0.860	0.0637	0.0526	0.0015	0.0076	0.0028	0.0122	0.0097	0.0188
129	May 22, 1924	0.782	0.0584	0.0478	0.0012	0.0100	0.0043	0.0150	0.0078	0.0097
153A	June 11, 1924	0.681	0.0578	0.0530	0.0019	0.0096	0.0028	0.0148	0.0103	0.0135
180	July 16, 1924	0.663	0.0612	0.0563	0.0014	0.0107	0.0014	0.0213	0.0096	0.0119
199	August 28, 1924	0.672	0.0571	0.0510	None	0.0097	0.0039	0.0135	0.0112	0.0127
215	October 7, 1924	0.543	0.0539	0.0480	None	0.0090	0.0052	0.0139	0.0099	0.0080
233A	November 11, 1924	0.443	0.0493	0.0437	None	0.0068	0.0071	0.0082	0.0107	0.0190
			As percentages of moisture free weight of material							
106	May 13, 1924	3.520	0.2300	0.1900	0.0055	0.0275	0.0101	0.0440	0.0350	0.0677
129	May 22, 1924	3.400	0.2540	0.2080	0.0050	0.0435	0.0185	0.0650	0.0340	0.0420
153A	June 11, 1924	1.980	0.1680	0.1540	0.0055	0.0280	0.0082	0.0430	0.0300	0.0393
180	July 16, 1924	1.926	0.1780	0.1637	0.0040	0.0312	0.0040	0.0620	0.0280	0.0345
199	August 28, 1924	1.740	0.1480	0.1320	None	0.0250	0.0100	0.0350	0.0290	0.0330
215	October 7, 1924	1.560	0.1550	0.1320	None	0.0260	0.0150	0.0400	0.0285	0.0230
233A	November 11, 1924	1.240	0.1380	0.1225	None	0.0190	0.0200	0.0230	0.0300	0.0305

ACCURACY AND LIMITS OF ERROR

Table IV gives the differences between duplicate determinations in certain of the series calculated as percentages of the mean readings.

TABLE IV

DIFFERENCES OBTAINED IN CERTAIN DUPLICATE DETERMINATIONS

SERIES NO.	TOTAL N	TOTAL WATER-SOLUBLE N	α-MONO-AMINO N	AMIDE N
	Per cent.	Per cent.	Per cent.	Per cent.
60	0.48	1.60	6.75	7.80
76	....	1.20	7.20	3.00
114	0.40	1.30	4.60	7.10
129	0.14	....	5.80	1.00
138	0.30	1.20	3.90	4.50

The total nitrogen shows a maximum difference of 0.48 per cent.; the water-soluble nitrogen of 1.60 per cent.; the α-mono-amino nitrogen of 7.20 per cent.; and the amide nitrogen of 7.80 per cent. All variations in the metabolism figures, therefore, can be regarded as significant.

Discussion and conclusions

TOTAL NITROGEN

TOTAL NITROGEN IN THE ONE YEAR BRANCH GROWTH.—It was pointed out in the third paper (7) that during the first growth of spring translocation of nitrogen from the one and two year branches to the young shoots is very marked. The effect of this demand is strikingly illustrated in the present investigation, as shown in fig. 1. The total nitrogen of the one year growth of the untreated tree drops during the period from April 28 to May 13 from 0.288 per cent. to 0.166 per cent. on the fresh weight basis, *i.e.*, from 0.720 per cent. to 0.480 per cent. on the moisture free basis. On May 13 the leaves contain as high as 0.860 per cent. nitrogen on the fresh weight basis and 3.520 per cent. on the moisture free basis.

The effect of the application of 10 pounds of $NaNO_3$ on April 20 to tree no. E–20 is distinctly evident in both the quantitative and qualitative relationships. After May 13, when the trees are in full bloom, the total nitrogen curves of the two trees take a different course. Basing observations on the moisture free basis (dy/dt), *i.e.*, the slope of the total nitrogen curves of the untreated tree is negative to the end of September; whereas in the treated tree (dy/dt), though fluctuating considerably, shows a net change of almost zero. In other words, from May 13 to the end of September there has been

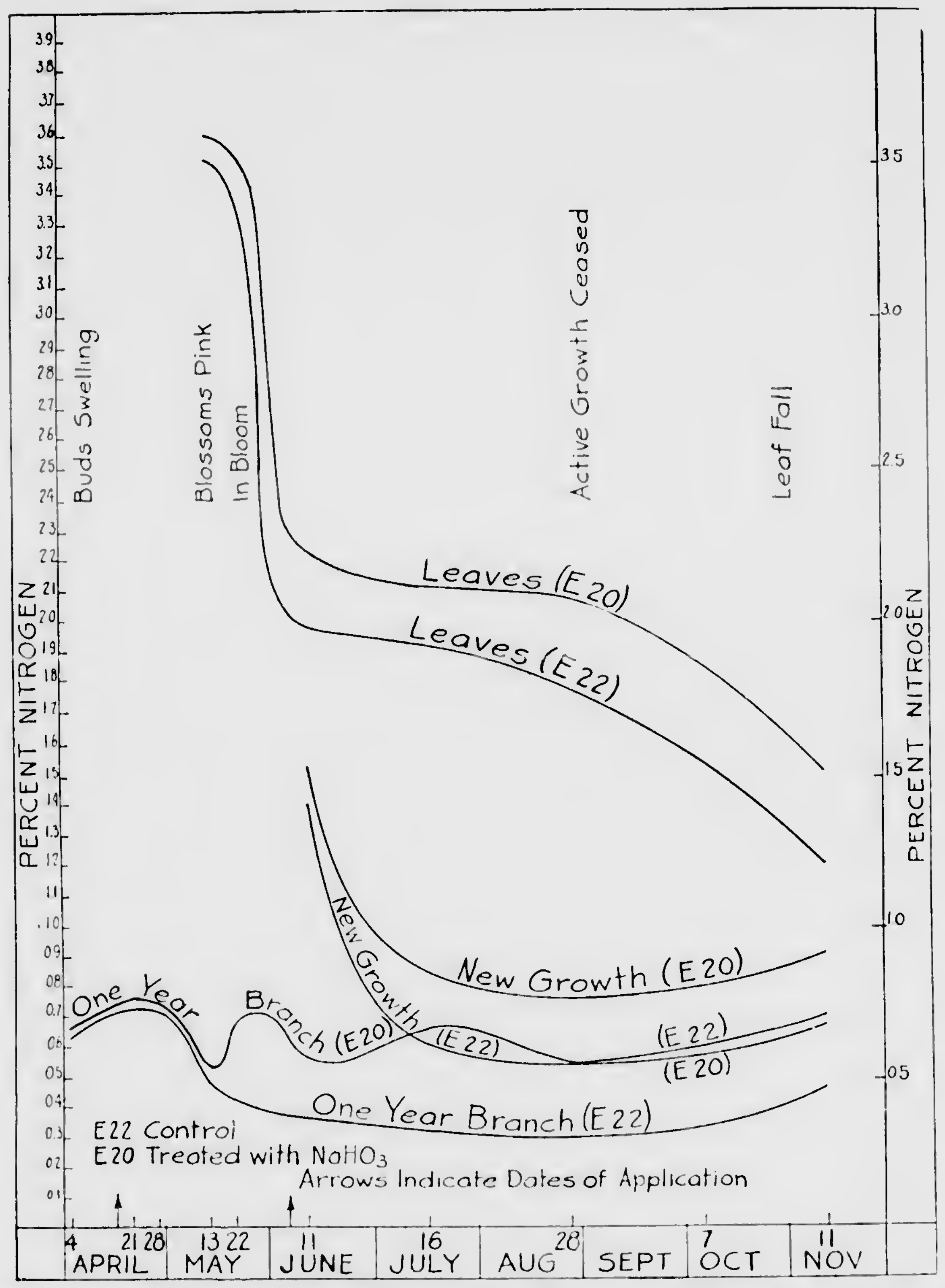

FIG. 1. Total nitrogen in the leaves, new (1924) branch growth and one year (1923) branch growth.

no net gain or loss in total nitrogen in the one year (1923) branch growth of the treated tree; whereas in the unfertilized tree the total nitrogen of the one year (1923) branch growth decreased 35 per cent. during the same period.

The increase in the total nitrogen content of the one year branch growth of tree no. E–20 continues until it is utilized by the demands of the developing shoots. An inspection of the curves shows that the second application (June 8) of 10 pounds of $NaNO_3$ to E–20 is absorbed more rapidly than the first application. These facts lend further support to the suggestion that the rate of absorption of a nutritive element is fundamental and probably one of the most important factors in soil fertility and plant nutrition problems.

The differences in total nitrogen content as a result of fertilization are again clearly indicated at the period when the trees enter the rest period. On April 4 the one year branch growth of the untreated tree has 0.299 per cent. nitrogen, but on November 11 only 0.208 per cent. on the fresh weight basis, *i.e.*, 0.635 per cent. and 0.437 per cent., respectively, on the moisture free basis; whereas the treated tree has 0.263 per cent. and 0.346 per cent., *i.e.*, 0.628 per cent. and 0.690 per cent. on the fresh and moisture free basis, respectively, on the respective dates. The results are clearly shown in table V.

TABLE V

COMPARISON OF THE PERCENTAGES OF TOTAL NITROGEN OF THE ONE YEAR BRANCH GROWTHS OF EACH TREE AT CERTAIN PERIODS

COLLECTING DATE	UNTREATED		TREATED	
	Fresh weight basis	Moisture free basis	Fresh weight basis	Moisture free basis
April 4, 1924	0.299	0.635	0.263	0.628
April 28, 1924	0.288	0.720	0.329	0.764
November 11, 1924	0.208	0.437	0.346	0.690

TOTAL NITROGEN OF THE NEW (1924) BRANCH GROWTH.—The differences in total nitrogen content of the new (1924) branch growth are also quite marked on the two trees. The untreated tree had a total nitrogen content of 0.379 per cent. on June 11 and of 0.300 per cent. on November 11 on the fresh weight basis, *i.e.*, 1.40 per cent. and 0.603 per cent., respectively, on the moisture free basis; whereas the treated tree had a total nitrogen content of 0.432 per cent. and 0.517 per cent. on the fresh weight basis, *i.e.*, 1.503 per cent. and 0.900 per cent., respectively, on the moisture free basis on these dates.

Table VI conveniently shows the effect of the $NaNO_3$ applications on the total nitrogen content of the new growth.

It has already been shown in the third paper that the results of applying 5 pounds of nitrogen are only just about sufficient to maintain nitrogen equilibrium under the conditions of that experiment. Considering, then, only the season's (1924) branch growth and the one year (1923) branch growth, the present results show that whereas the untreated tree will start the next season on a much lower nitrogen plane than in the previous season, the treated tree has slightly more than maintained equilibrium. This appears to be in accordance with the practical results obtained in this Experiment Station, indicating that 10-pound applications of $NaNO_3$ per tree may be practicable economically.

TABLE VI

COMPARISON OF THE PERCENTAGES OF TOTAL NITROGEN OF THE NEW (1924) BRANCH GROWTHS OF EACH TREE AT CERTAIN PERIODS

COLLECTING DATE	UNTREATED		TREATED	
	Fresh weight basis	Moisture free basis	Fresh weight basis	Moisture free basis
June 11, 1924	0.379	1.400	0.432	1.503
November 11, 1924	0.300	0.604	0.517	0.900

TOTAL NITROGEN IN THE LEAVES.—Table VII shows the effect of fertilization on the total nitrogen content of the leaves. The effect on the leaves is more clearly indicated in the diminution in concentration of nitrogen as indicated by the much lower figures for the untreated tree on the fresh weight basis than in the absolute quantities.

TABLE VII

COMPARISON OF THE PERCENTAGES OF TOTAL NITROGEN OF THE LEAVES OF EACH TREE AT CERTAIN PERIODS

COLLECTING DATE	UNTREATED		TREATED	
	Fresh weight basis	Moisture free basis	Fresh weight basis	Moisture free basis
May 13, 1924	0.860	3.520	0.830	3.610
November 11, 1924	0.443	1.240	0.677	1.550

Although the character of the curves is the same, there are decided quantitative differences. On May 13 the total nitrogen content of the young leaves of the untreated tree is 0.860 per cent. and of the treated tree 0.830 per cent. (fresh weight basis), or 3.520 per cent. and 3.610 per cent. (moisture free basis), respectively. At the end of the period of chlorophyll degeneration, about the time of leaf fall, the untreated tree contained 0.443 per cent. and the treated tree 0.677 per cent. (fresh weight basis), or 1.240 per cent. and 1.550 per cent. (moisture free basis), respectively.

The same fall in total nitrogen content of the leaves from the time of bud opening to the middle of June, observed in the previous investigation and described in the third paper of the series, takes place in both trees. This corresponds to the period of most rapid growth.

THE NITROGEN DISTRIBUTION

THE PARTITION OF NITROGEN IN THE LEAVES.—The marked differences in the nitrogen metabolism of the two trees is very evident from an inspection

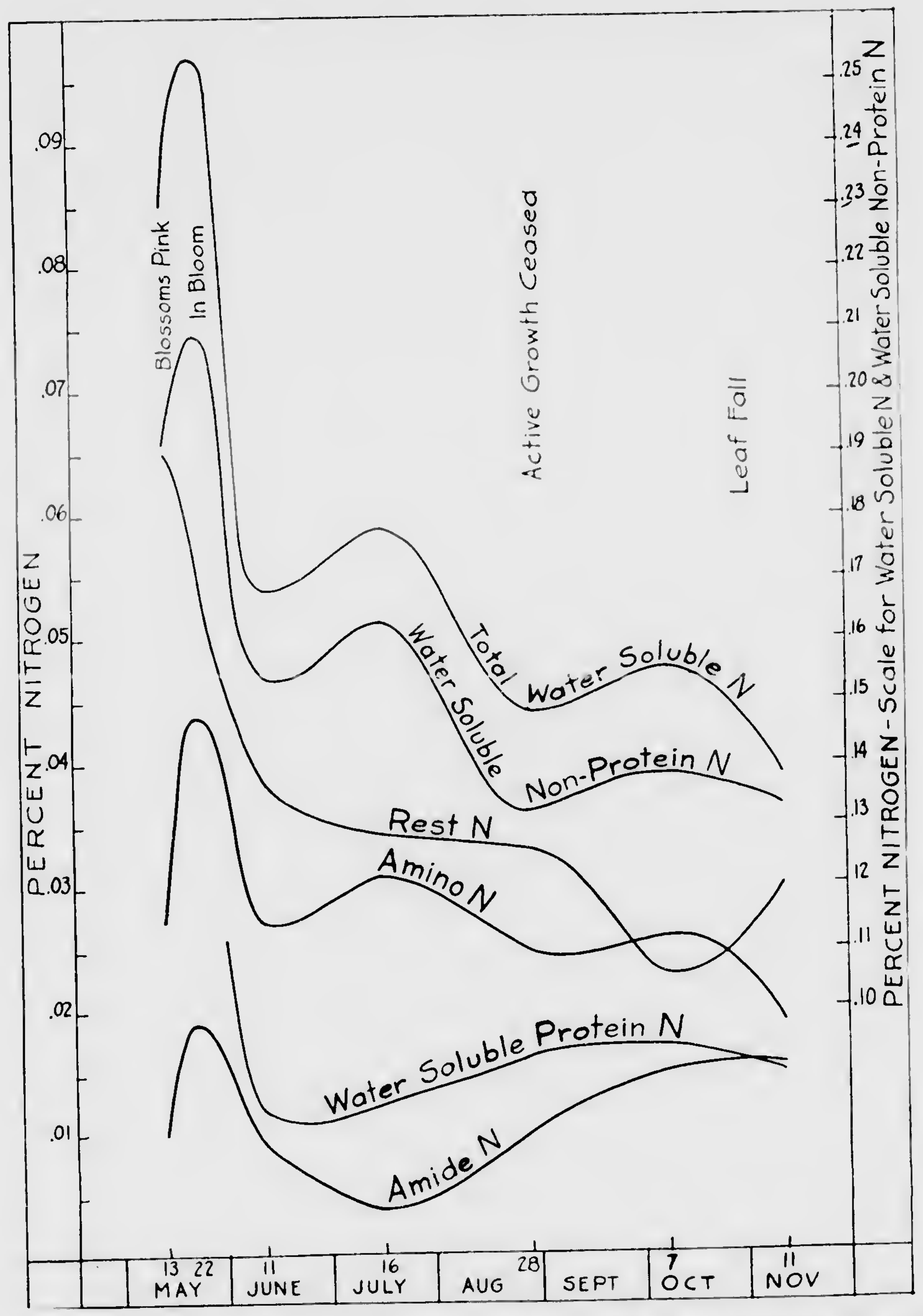

FIG. 2. Total water-soluble N, water-soluble protein N, non-protein N, amino N, amide N and "rest" N in the leaves of the untreated tree (no. E–22).

of figs. 2 and 3. The analytical results are given in table III. The most striking differences are in the total water-soluble nitrogen, the non-protein nitrogen, the amino nitrogen, and the "rest" nitrogen. The effect of the second application of $NaNO_3$ on June 8 results in a large increase of total water-soluble and non-protein nitrogen. Thus, in the treated tree the increase in concentration (as indicated by the fresh weight results) of water-soluble N and non-protein N from June 11 to July 16 amounts to 43 and 16 per cent., respectively, but during the same period the total water-soluble and non-protein nitrogen of the leaves of the untreated tree show an increase of only 6 and 8 per cent., respectively. The earlier investigations show a similar fluctuation, occurring, however, a month later. That the increase at this period in the untreated tree can be due solely to increased nitrification of the soil is quite clear, for fig. 4 shows that the one year (1923) branch growth increases in total water-soluble nitrogen also.

The course followed by the amino and amide nitrogen in the leaves calls for no special comment. The curves are similar to those described in the earlier investigations (**7**). It is to be noted that they are much higher in the fertilized tree as a result of the $NaNO_3$ treatment.

The "rest" nitrogen compounds represent, both in absolute magnitude and in their metabolic gyrations, an extremely important group in that the evidence indicates that these unclassified compounds form an important link in the degradation of proteins (**7**). The fluctuations of this fraction in the two trees show more remarkable differences than any of the other fractions.

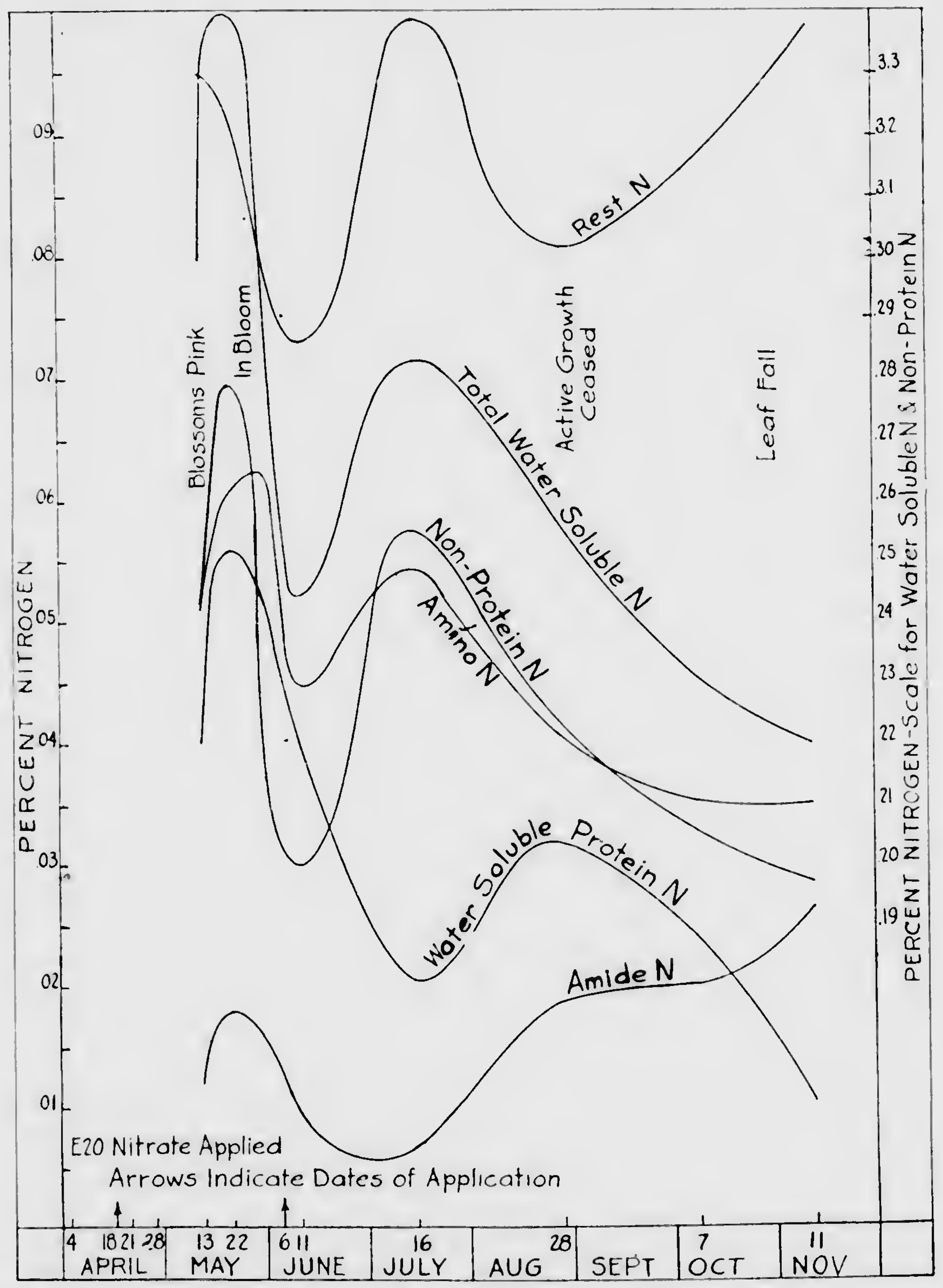

FIG. 3. Total water-soluble N, water-soluble protein N, non-protein N, amino N, amide N and "rest" N in the leaves of the treated tree (no. E–20).

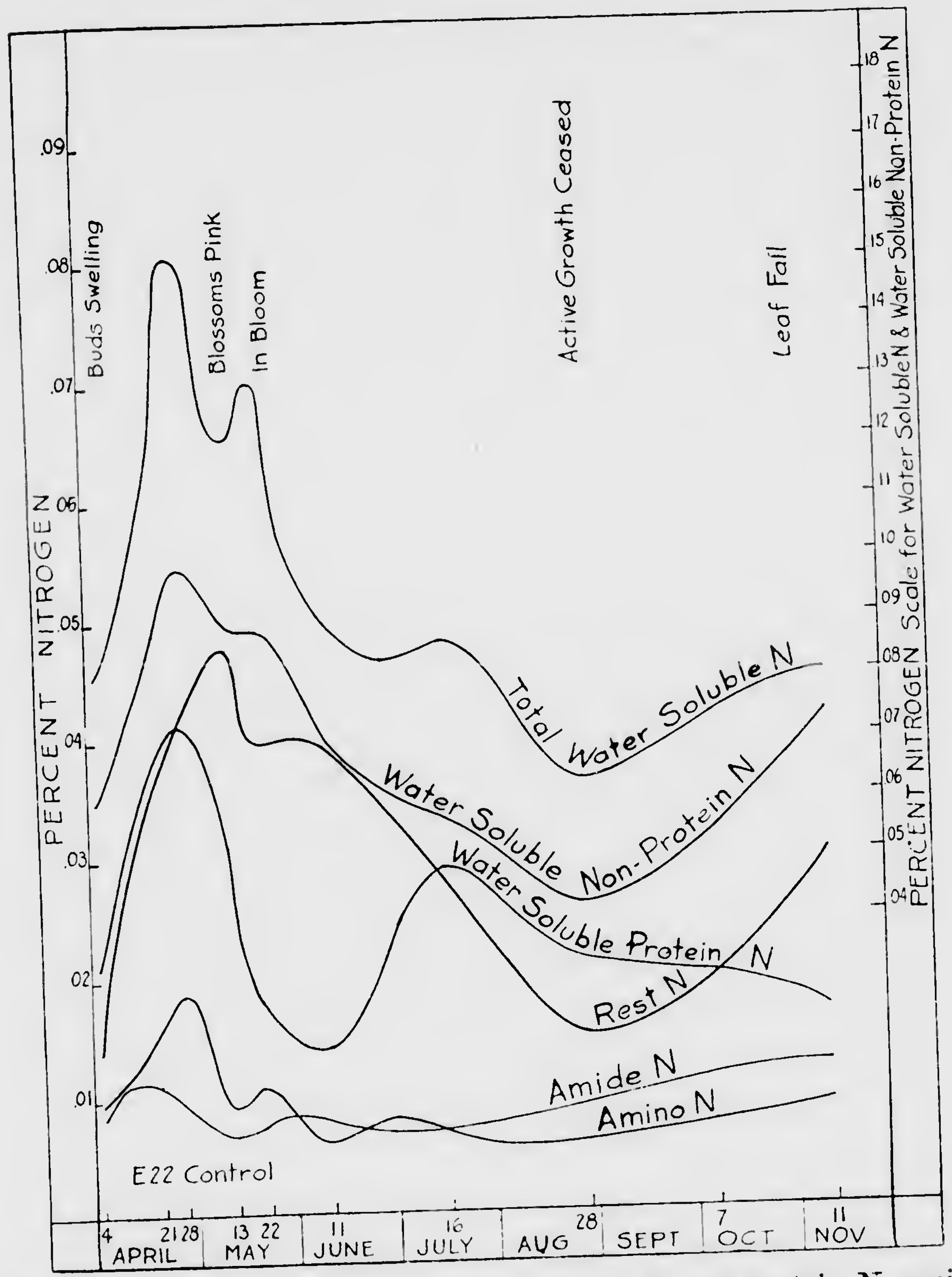

FIG. 4. Total water-soluble N, water-soluble protein N, non-protein N, amino N, amide N and "rest" N in the one year (1923) branch growth of the untreated tree (no. E–22).

The positive slope of the "rest" nitrogen curve in fig. 3 just after the application of nitrate to the treated tree on June 8 is a characteristic of this tree only; the leaves of the untreated tree do not show it. It is hoped to be able to isolate and identify some of these unclassified nitrogen compounds later.

THE PARTITION OF NITROGEN IN THE ONE YEAR (1923) BRANCH GROWTH.—The conclusions drawn in the earlier investigations are, in general, confirmed in this investigation; and it will, therefore, be unnecessary to discuss them in detail again. The visual presentation by means of figs. 4 and 5 shows very clearly the differences between the treated and untreated trees.

The influence of the sodium nitrate addition on the nitrogen partition on the one year (1923) branch growth of tree no. E–20 on April 20 and

June 8 is very marked. From a comparison of the cycle curves (figs. 4 and 5) it is readily seen that the period of absorption is characterized by a marked increase in the total water-soluble and the non-protein nitrogen with concomitant increases in the amino, amide and basic nitrogen of tree E–20 (treated) in comparison with tree E–22 (untreated).

On April 21 the percentages of total water-soluble nitrogen in the one year (1923) branch growth of trees E–20 (treated) and E–22 (untreated) are 0.0463 and 0.0520 per cent., respectively, but three weeks later the quantities present are 0.0789 and 0.0385 per cent., respectively—an increase of 85 per cent. and a decrease of 30 per cent., respectively. Again, at the last sampling, November 11, tree no. E–20 (treated) has 0.0852 per cent., but tree no. E–22 (untreated) has only 0.0432 per cent. of water-soluble nitrogen. The non-protein nitrogen results of the trees parallel these figures, *viz.*, 0.0380 and 0.0389 per cent. on April 21 and 0.0429 and 0.0375 per cent. three weeks later.

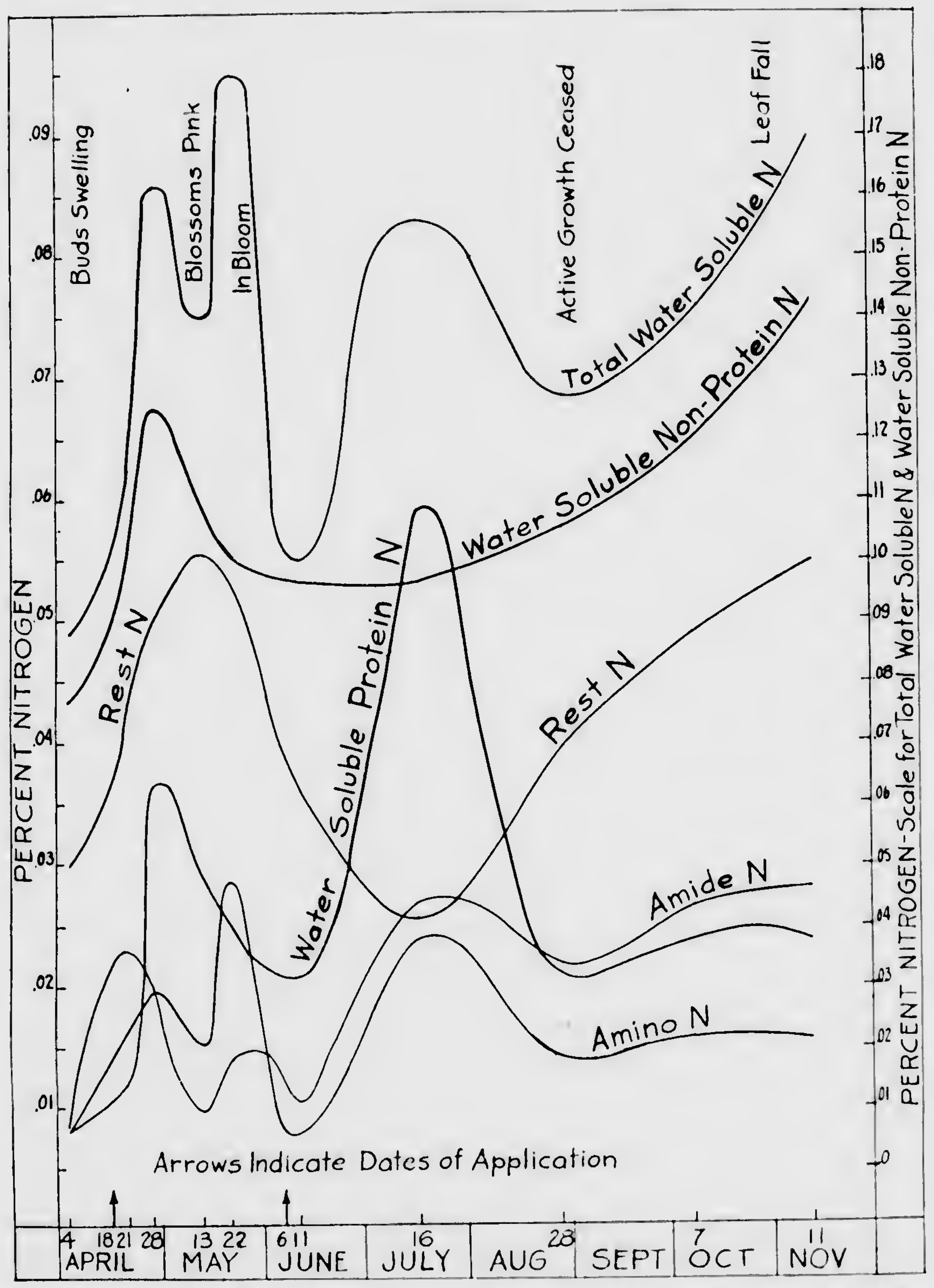

FIG. 5. Total water-soluble N, water-soluble protein N, non-protein N, amino N, amide N and "rest" N in the one year (1923) branch growth of the treated tree (no. E–20).

The mono-amino and amide nitrogen curves, too, are interesting. Up to May 10 (about three weeks after the first application of $NaNO_3$ to E–20) the curves for amino N show little differences in the two trees. About this date, however, the mono-amino and amide nitrogen begin to increase rapidly in the treated tree. The same phenomena are observed after the second application of $NaNO_3$ on June 8. As already pointed out, at this time of the year absorption of the $NaNO_3$ and its transformation to $-NH_2$ and $-CO.NH_2$ groups is more rapid, for the effect is noticeable in the leaves, one year branch growth and new growth within ten days of the second application.

The importance of the "rest" nitrogen compounds both in magnitude and in their metabolic gyrations is again clearly indicated in the one year (1923) branch growth. Their significance is more or less a mystery, but an inspection of the graphs will readily confirm the conclusions previously drawn (7) where it was suggested that they must take part in the degradation of proteins.

THE PARTITION OF NITROGEN IN THE NEW (1924) BRANCH GROWTH.—The differences in the nitrogen partition in the new (1924) branch growth are more marked than in the one year (1923) branch growth. The behavior of the treated tree (E–20) with respect to the total water-soluble and non-protein nitrogen cycle corresponds to the results obtained in the earlier investigations (7). The main point of interest is the steep positive slope as shown in fig. 7, about a week after the second application of $NaNO_3$ (June 8), and the very large increase again in these constituents during the fall storage of nitrogen.

A complete interpretation of the total water-soluble and non-protein nitrogen cycles (fig. 6) of the untreated tree is more difficult. It is apparent that the concentrations of nitrogen in both fractions are much lower in the check tree than in the nitrogen treated tree and that these differences are associated with differences in vigor.

The mono-amino acid and amide nitrogen cycles in the new (1924) branch growth of the two trees show the characteristic differences already observed in the one year (1923) branch growth, *e.g.*, the effect of the second application of $NaNO_3$ on June 8 to tree E–20 is very evident from the marked increase in amino N and the "rest" N compounds. The large storage as amide N in the new branch growth as a result of fertilization is very marked; thus, on November 11 the amide nitrogen in E–20 is 0.0201 per cent. and 0.0350 per cent., but in E–22 only 0.0087 per cent. and 0.0180 per

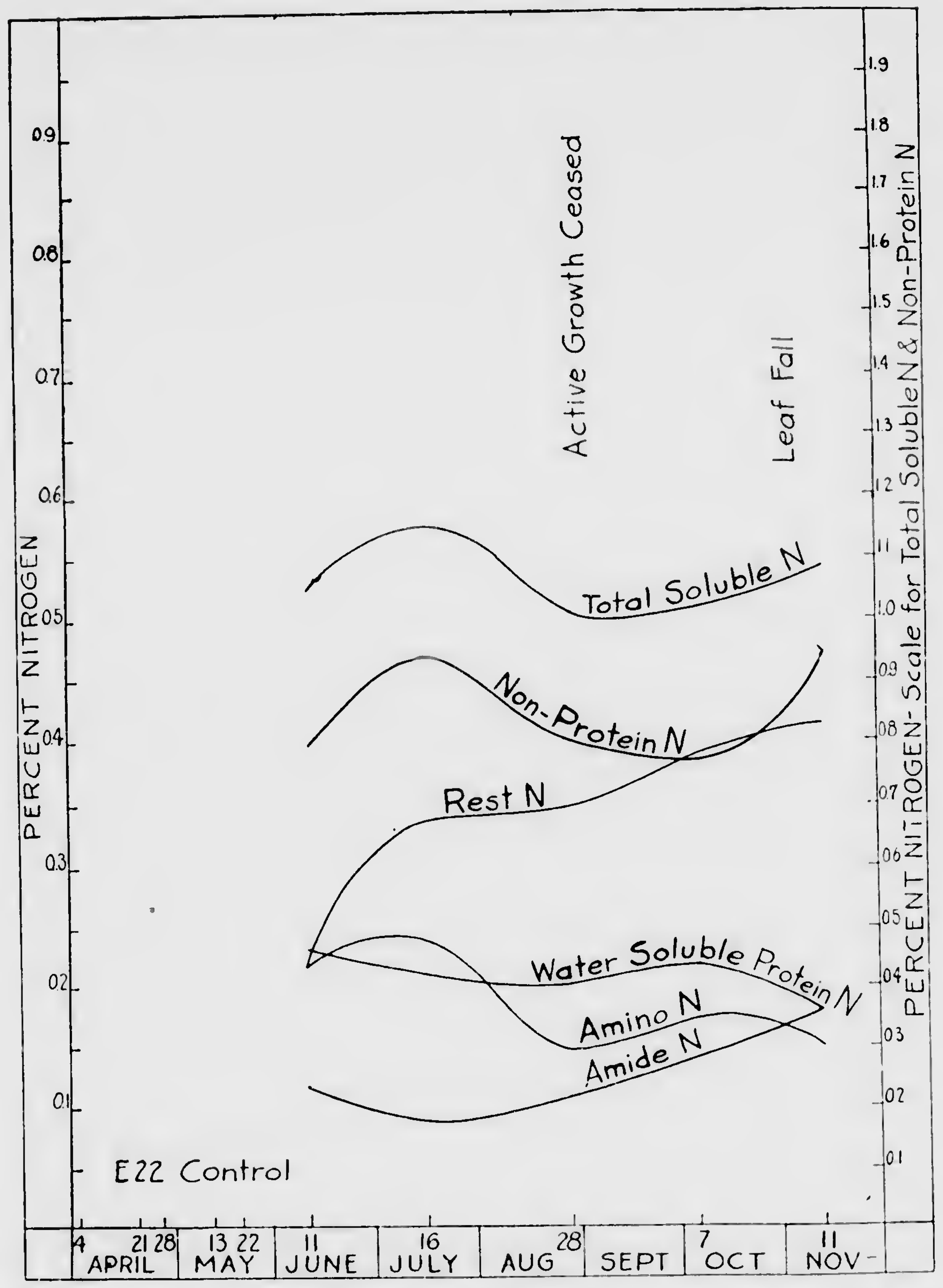

Fig. 6. Total water-soluble N, water-soluble protein N, non-protein N, amino N, amide N and "rest" N in the new (1924) branch growth of the untreated tree (no. E–22).

cent. on the fresh and moisture free basis, respectively, and the mono-amino N 0.0281 per cent. and 0.0490 per cent. and 0.0073 per cent. and 0.0150 per cent. on the fresh and moisture free basis, respectively.

Summary and conclusions

These studies are an extension of the partition investigations reported in the third paper of the series (7) to two Stayman Winesap trees of the same age, growing on sod in a homogenous soil. One of these trees was fertilized with $NaNO_3$ and the other left unfertilized. In general, the present findings confirm the conclusions drawn from the previous studies.

The present investigations have shown that:

1. The total nitrogen of the leaves and shoot growth vary with growth. During the early growth of spring the demand of the young shoots upon the nitrogen of the one year (1923) branch growth is very marked. This is especially shown in the untreated tree, E–22.
2. The effect of the two 10-pound applications of sodium nitrate on April 20, 1924, and June 8, 1924, on the total nitrogen content of the two trees may be thus summarized:
   (a) The total nitrogen content of the one year (1923) branch growth of the fertilized tree from the period of full bloom to the end of September increased 10 per cent., but the total nitrogen of the

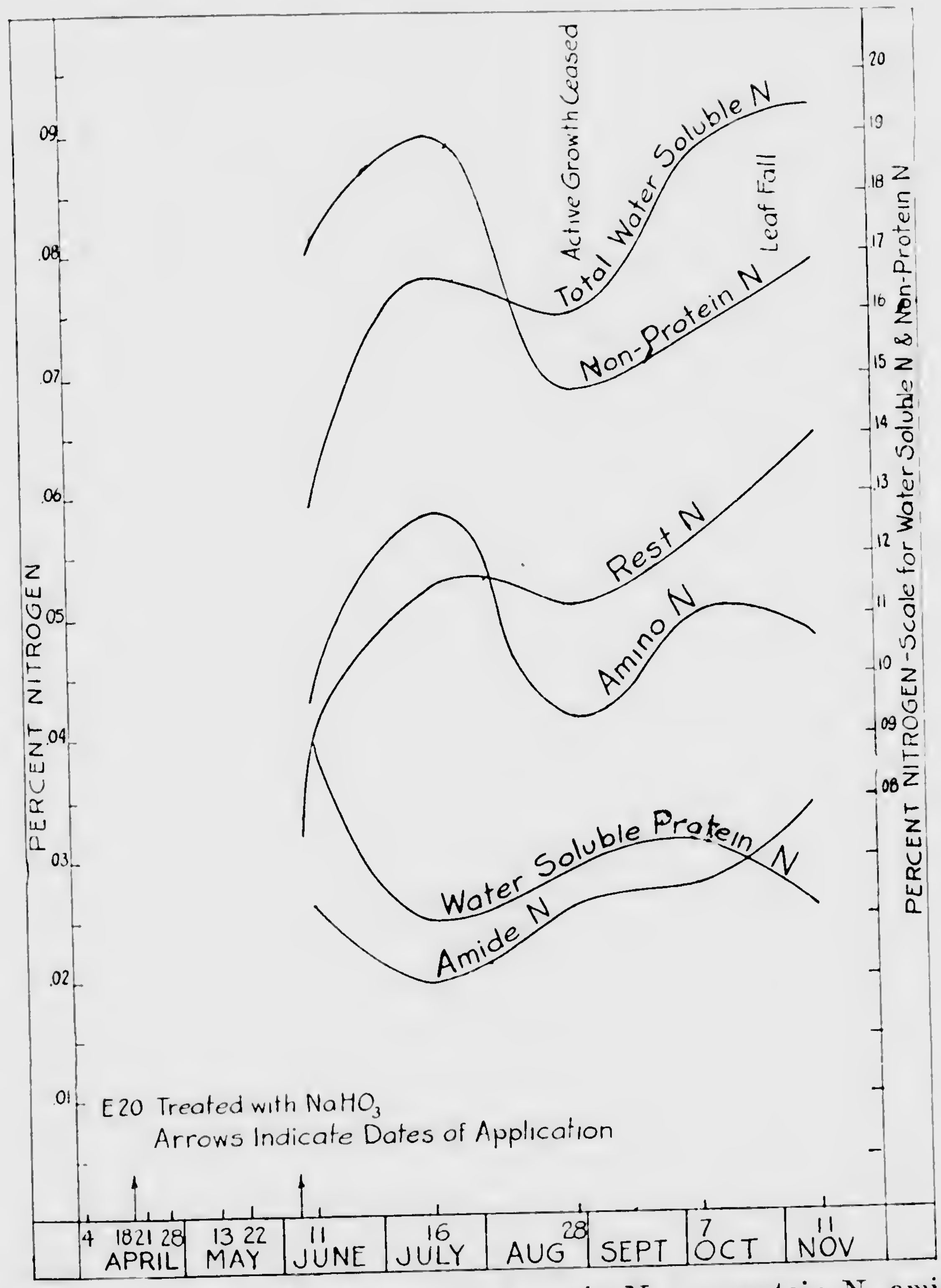

Fig. 7. Total water-soluble N, water-soluble protein N, non-protein N, amino N, amide N and "rest" N in the new (1924) branch growth of the treated tree (no. E–20).

one year (1923) branch growth of the unfertilized tree decreased 35 per cent. during this period.

(b) From June 11 to November 11 the total nitrogen of the new growth of the treated tree (E–20) decreased 40 per cent., but that of the untreated tree (E–22) decreased 57 per cent.

(c) The leaves of the untreated tree are consistently lower than in the treated tree.

A much lower plane of nitrogen metabolism is thus indicated in the unfertilized tree.

3. The period of most rapid absorption of the NO_3 ion is characterized by a large increase in the total water-soluble and non-protein nitrogen, concomitant with which there is an increase in the amino, amide, basic, and "rest" nitrogen fractions of the fertilized as compared with the unfertilized tree in the leaves, the one year (1923) branch growth and the new (1924) branch growth.
4. From the course of the curves for total water-soluble and non-protein nitrogen or amino N of the fertilized as compared with the unfertilized tree, it can be deduced that the first application of sodium nitrate on April 20 took three weeks to reach the one year branches. The same curves show that the absorption and translocation of the NO_3 ions of the second application on June 6 is much more rapid, taking about one week only to get to the more metabolically active parts of the tree.
5. The total water-soluble nitrogen and non-protein nitrogen is decidedly higher in the metabolically active parts of the treated tree as compared with the untreated tree throughout the whole cycle. These differences correspond with the relative vigor of the trees and must be associated with differences in enzymatic activity. Taken in conjunction with the close parallelism of the amino-acid fraction with the total water-soluble and non-protein fractions (7), these findings suggest a possible simplification of the nitrogen distribution problem in practical horticulture.
6. Terminal growth was decidedly greater in the fertilized than in the unfertilized tree.
7. Nitrogen is stored in the fall as amide N and as "rest" N compounds. The storage of these compounds is much higher in the treated tree than in the untreated tree. This fact is of extreme importance in connection with the demands of the young shoots in the spring. This is true of both the one year (1923) branch growth and the new (1924) branch growth.
8. The "rest" nitrogen compounds form an important connecting link in the synthesis and degradation of proteins.

The writer desires to thank Mr. C. A. Kern for the determinations of the hygroscopic and imbibitional water and Miss Ethel Gingrich for assistance in calculating and checking the computations.

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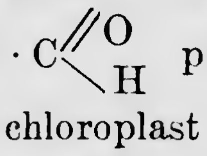
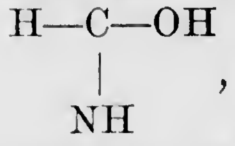
THE SEAT OF FORMATION OF AMINO ACIDS IN PYRUS MALUS L.[1]

ALTHOUGH it has been established that nitrates can be reduced to nitrites in the roots and stems of plants, the possibility that the reduction of nitrites to form α-amino acids as an intermediate stage in the synthesis of proteins in plants could also take place in the cells of the roots and stems has not generally been accepted. Emmerling[2] indicated that in certain soils amino acids might be formed from nitrates in the roots and stems of some plants. However, Sachs (1865), Sorokin (1870), Pagnourd (1879) and Schimper[3] (1885) supported the photochemical theory of the formation of amino acids and proteins. They considered the chloroplast of the cells of the leaves maintained with a continuous supply of carbohydrates as being specially adapted to carry on the synthesis of proteins from ammonium salts and nitrates supplied to them by the conducting cells. Moreover, the rapid consumption of nitrates in the leaves is offered as the reason for the lower nitrate content in these tissues than in the roots and stems of some plants.

This view that the synthesis of amino acids can take place in the leaves only has been especially emphasized more recently by Baly and his colleagues[4] from their experiments on the reduction of nitrates *in vitro*. It is maintained by these investigators that the activated formaldehyde  $H \cdot C \begin{smallmatrix} /\!\!/ O \\ \diagdown H \end{smallmatrix}$ produced photosynthetically in the living chloroplast cells reacts upon the first product of reduction (the nitrous acid salt—KNO_2) giving rise to formhydroxamic acid  $\begin{matrix} H—C—OH \\ \,\,\,| \\ NH \end{matrix}$, the formation of which was first established by Baudisch[5] by exposing a solution KNO_2 and methyl alcohol to ultra-violet light. This latter is assumed by Baly and his colleagues to react with another molecule of activated formaldehyde, giving rise to numerous nitrogen compounds such as, for example, the α-amino acid—glycine. It follows, they claim, that the synthesis of nitrogen compounds ***must*** be restricted to the leaves. Although these papers have attracted considerable attention, plant physiologists have necessarily been cautious in accepting the conclusions drawn.

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[1] Published by permission of the Director of the Agricultural Experiment Station as Technical Paper No. 425. Contribution No. 28 of the Department of Agricultural and Biological Chemistry.

[2] Emmerling, A., numerous papers in Landw. Ver. Stat. (1880–1898).

[3] See Czapek, F., Biochem. der Pflanzen 2: pp. 296–301 (1920) for a résumé.

Thus, Dr. Eckerson[6] has shown that the hypothesis propounded by Baly *et al* that nitrates are reduced in the light by activated formaldehyde in green leaves is inapplicable to the results of her experiments on tomato plants having a high C : N ratio, since, in this case, when nitrates are fed, it is the fructose and glucose that are oxidized, accompanied by an hydrolysis of starch as the hexoses are used up in the formation of amino acids and a portion possibly in

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[4] Baly, E. C., Heilbron, I. M., and Hudson, D. P., *J. Chem. Soc.* (Lond.) 121: pp. 1078–1088 (1922).

[5] Baudisch, Oskar, Ber. der Deut. Chem. Ges. 44: pp. 1009–1013 (1911).

[6] Eckerson, Sophia, *Bot. Gaz.*, 77: pp. 377–390 (1924).

increased respiration. Suzuki[7] also obtained strong nitrate tests with barley plants fed nitrate only, the nitrate disappearing when sugar was added. The nature of the active material is unknown. Anderson[8] has postulated the presence of a reducing substance resembling the atite of Haas and Hill.[9]

During the investigations of the writer, extending over the past four years, on the nitrogen metabolism of Pyrus Malus, in which the partition of nitrogen has been studied in the various parts throughout a year's cycle, positive tests were found for nitrates (or nitrites) in one tissue only and this at just one period of the year, *viz.,* in the leaf buds just as they were opening. This work was carried out on mature and seedling apple trees receiving heavy applications of sodium nitrate at regular intervals throughout the vegetative period, by means of microchemical tests on sections of the leaves, tips of stems and one and two-year old branches with diphenylamine reagent,[10] "G" salt,[11] and the Griess-Ilosvay Reagent,[10] and also numerous *quantitative* tests[12, 13, 14] on both the dialyzed and undialyzed sap, preserved under toluene, and on aqueous alcoholic extracts of various tissues during the vegetative cycle. The fine roots gave nitrate reactions throughout the season; whereas in the main roots the reaction was much feebler and, as already stated, the tests were negative in the aerial parts except in the buds as they were opening. Correspondingly, quantitative tests for amino acids were always higher in the roots than in the aerial parts.

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[7] Suzuki, U., Bull. Coll. Agr. Imp. Univ. Tokyo 3: pp. 488–507 (1898).

[8] Anderson, V. L., Ann. Bot., 38: pp. 699–706 (1924).

[9] Haas, P., and Hill, T. G., *Biochem. J.*, 17: pp. 671–682 (1923).

[10] Eckerson, Sophia (*loc. cit.*, see p. 379).

[11] Nixon, T. G., *Chem. News*, 126: p. 261 (1923).

[12] Withers, W. A., Ray, B. J., *J. Am. Chem. Soc.*, 33: p. 708 (1911).

[13] Strowd, W. H., *Soil Sci.*, 10: pp. 333–342 (1920).

[14] Gallagher, P. H., *J. Agr. Sci.*, 13: pp. 61–63 (1923).

These results are in accord with the recent work of Dr. Eckerson,[15] who found that the reducing power of extracts from various parts of apple trees collected last September and November showed decided differences. The fine roots were very high in reducing activity, the buds less active and the bark of first and second year twigs had very little reducing power.

From the foregoing, it can scarcely be doubted that in this species the reduction of nitrates to amino acids takes place for the most part in the roots. Although experiments *in vitro* may be valuable in suggesting types of reactions that may occur in the cells of plants or animals, the extension of the results of such experiments as indicating the actual conditions existing to the processes *in vivo* should be made with caution. The internal conditions existing in the plant at any one time may bring about unlike chemical reactions to accomplish the synthesis of α-amino acids and the different plant species may not carry out these syntheses in the same way. These investigations do not throw any light on the mechanism of the formation of amino acids in this plant and any suggestions offered at present would be purely hypothetical.

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[15] Eckerson, Sophia. Private communication.

Pa.-25

THE RELATION BETWEEN THE VITAMIN B CONTENT OF THE FEED EATEN AND OF THE MILK PRODUCED

BY

S. I. BECHDEL AND HANNAH E. HONEYWELL
(Contribution from the Pennsylvania State College)

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THE RELATION BETWEEN THE VITAMIN B CONTENT OF THE FEED EATEN AND OF THE MILK PRODUCED[1]

By S. I. BECHDEL, *Department of Dairy Husbandry*, and HANNAH E. HONEYWELL, *Department of Agricultural and Biological Chemistry, the Pennsylvania State College*[2]

INTRODUCTION

It has been clearly demonstrated by investigators in England (*1*, *3*)[3] and America (*5*, *7*, *8*, *9*, *10*) that the degree of potency of vitamins A and C in milk is largely dependent on the diet of the cow. The greater part of the evidence has been obtained from comparisons of milk produced on summer pasture with milk produced on the usual winter ration, consisting of silage, grain, and hay.

It would appear reasonable to expect also that the vitamin B growth-promoting factor in milk would be dependent on the diet of the cow. Evidence obtained by several investigators does not bear out such assumption in all instances. Osborne and Mendel (*11*) have reported that from the standpoint of vitamin B potency milk from pasture-fed cows was not superior to that from cows receiving winter rations. Later investigations were conducted by Kennedy and Dutcher (*10*), Hughes, Fitch, and Cave (*9*), who concluded that the presence of vitamin B in milk was dependent on the presence of this factor in the diet of the cow. More recently Hart (*6*) has expressed the opinion that milk produced by cows on pasture is no richer in vitamin B than that from cows fed the usual winter ration of grain, hay, and silage. It has also been reported that it is not possible through the feeding of excessive quantities of wheat germ to increase the vitamin B content of cow's milk (*12, p. 18*). Just recently Bechdel, Eckles, and Palmer (*2*) concluded that the feeding of cows on a ration deficient in vitamin B slightly reduced the vitamin B content of the milk. As the ration fed the cows used in this investigation was unpalatable and was not eaten in amounts sufficient for satisfactory nutrition, the authors were reluctant to consider the results as being highly significant.

For the past three years one of the authors has been in charge of an investigation to determine the vitamin B requirements of calves. Conclusive evidence has been obtained to show that a calf will grow normally to maturity, and produce normal offspring on a ration that carries an insufficient amount of vitamin B to support growth and well-being in rats (*2*). Since the animals used in these investigations had been fed on such a ration for over two years at the time of freshening, an excellent opportunity was afforded

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[2] The authors wish to acknowledge their appreciation of the helpful advice received from Prof. R. A. Dutcher in planning and carrying out the experiment.

[3] Reference is made by number (italic) to "Literature cited," p. 287.

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for a study of the vitamin B content of milk which was produced on a ration definitely known to be deficient in vitamin B.

In all of the investigations previously carried out as cited above, the vitamin B content of the ration fed to the cows was not definitely determined through rat-feeding trials. Furthermore, in the previous investigations, the deficient ration was not fed for such a prolonged period of time before the cows began producing milk.

EXPERIMENTAL PROCEDURE

The object of the present study was to determine through laboratory feeding trials with rats, the vitamin B potency of milk produced by three of the vitamin B growth experimental animals (*2*), Nos. 1168, 1169, and 1170. Two of these cows, Nos. 1169 and 1170, had received the ration deficient in vitamin B (found so by 11 rat-feeding trials) throughout their growth and reproductive careers, a period of about 750 days. The other cow, No. 1168, had received a vitamin B supplement to her ration for a part of the time during her growth period (*2, p. 424*). Continued feeding of this vitamin B supplement (marmite yeast) was found unnecessary for growth and well-being, and it was dropped from the ration almost 300 days before she freshened.

Animal No. 1169 freshened December 18, 1925, and No. 1168 a week later. Two weeks after the latter had freshened, about 1 quarts of her milk and 30 quarts from No. 1169 were saved from 4 successive milkings for the experiments. The milk from these heifers was mixed, poured in sterile containers, sealed, and frozen solid. A refrigerator maintained at about 10° F. below zero was used for storing the milk until it was needed for experimental feeding.

The milk was all collected at one time for the reason that there was some doubt as to whether the heifers would be able to lactate on the experimental ration for any great length of time. It was fortunate that such provision was made, since it developed a few days later that the experimental ration was inadequate to meet the heavy demands of lactation. Subsequent investigations, the details of which will be submitted in a later paper, established definite proof that the apparent deficiency was not due to an inadequate supply of vitamin B. Since vitamin B storage has not been found in other species (*13*), it would have been very interesting to study this phase of the problem on cattle. The authors appreciated, however, that the possibility of vitamin B storage in these heifers was almost negligible, and also that the fact that the milk was obtained early in lactation would not materially depreciate the value of the data.

Animal No. 1170 freshened February 6, 1926, after it had been fed the deficient ration 776 days. Her milk was sampled and stored for experimental feeding as described above.

The experimental ration fed to the heifers was made up of dried sugar-beet pulp as the sole source of roughage and the following concentrates: Corn gluten, pearled hominy from white corn, polished rice, cornstarch, imported casein (Argentine), cane sugar, bone meal, and a mineral mixture. Cod-liver oil was fed daily to supply fat soluble vitamins.[4]

The laboratory methods previously employed for vitamin studies by Dutcher and his associates (*4*) were used in this investigation. A vitamin B free basal ration (*4, p. 383*) was fed in connection with the milk, as the sole source of vitamin B supply. Twelve cubic centimeters of milk from cows receiving good winter rations when fed in this way had been found necessary to support normal growth. The experiments were planned, therefore, with five groups of rats in order to study daily allowances per rat of 8, 10, 12, 16, and 20 c. c. of milk. Twelve rats were allotted to each group at the beginning of the experiment, and the mixed milk from cows Nos. 1168 and 1169 was fed to them. Beginning with the ninth week of feeding, all of the rats but the group allowed 20 c. c. of milk daily were fed milk from heifer No. 1170, as the remaining supply from the other heifers was inadequate to complete the experiment.

The rats were put on experiment at an average age of 24 days. Their average initial weight was 37 gm. In all of the work individual cages with screen bottoms (3 meshes to 1 inch) were used, a practice which has been rigidly carried out in this laboratory for over three years. Iodine was fed in the drinking water. The milk was fed in a container separate from the basal ration, and two drops of cod-liver oil were added to the milk daily to insure an adequate supply of vitamins A and D.

DISCUSSION

The experimental data were averaged and put in graphic form as presented in Figure 1. The groups fed 8 and 10 c. c. of milk (curves A and B) deviate markedly from the normal. None of the individual rats in the group allowed 8 c. c. were normal in size at the end of 91 days of feeding, although every individual continued to gain in weight during the entire feeding period. Similar comment may be made also concerning the group allowed 10 c. c. of milk, except that one individual was normal in size at the end of the experiment. There was apparently no tendency toward pathological conditions in any of the rats of the two groups above mentioned or of the groups fed larger quantities of milk.

The group receiving 12 c. c. of milk (curve C or curve H) made much better growth than those receiving less. Nine rats, or 50 per cent of this group, were of normal or larger size at the end of 84 days of feeding.

When the feeding of milk from heifer No. 1170 was started as mentioned above, an additional group of six rats was put on an experiment in which 12 c. c. of milk was added to the basal ration. This was done in order to determine whether the growth of the young, more rapidly growing animals would indicate any difference in the milk from another cow on the same diet. No detectable differences in the growth of any of the groups of rats was observed when the milk was changed. However, the growth of the additional six animals conformed closely to that of the rats from the same stock that were fed by Dutcher, Francis, and Combs (*4*) on 12 c. c. of raw milk from the college herd. For convenience of study, a copy of the growth curve on the latter data has been placed on the chart (curve F) beside those of the two groups of rats fed 12 c. c. of raw milk in this experiment (curves G and H). (Curve F copied from Dutcher, Francis, and Combs.)

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[4] Details concerning the ration and feeding technic may be found in the Journal of Dairy Science (*2, p. 417*).

In order to have enough animals of one age and weight to start the five groups at one time, the rats used were purchased from a supply company. It so happened that they were of a strain of a somewhat lighter weight than the laboratory stock. It is entirely probable that their growth (curve H) is as near normal for their weight as that of the Pennsylvania State Stock (curve G). From the evidence presented by this additional group in connection with that of the original 12 rats, it is apparent that 12 c. c. of milk from cows on a ration deficient in vitamin B carried as much vitamin B as 12 c. c. of milk from cows receiving good winter rations.

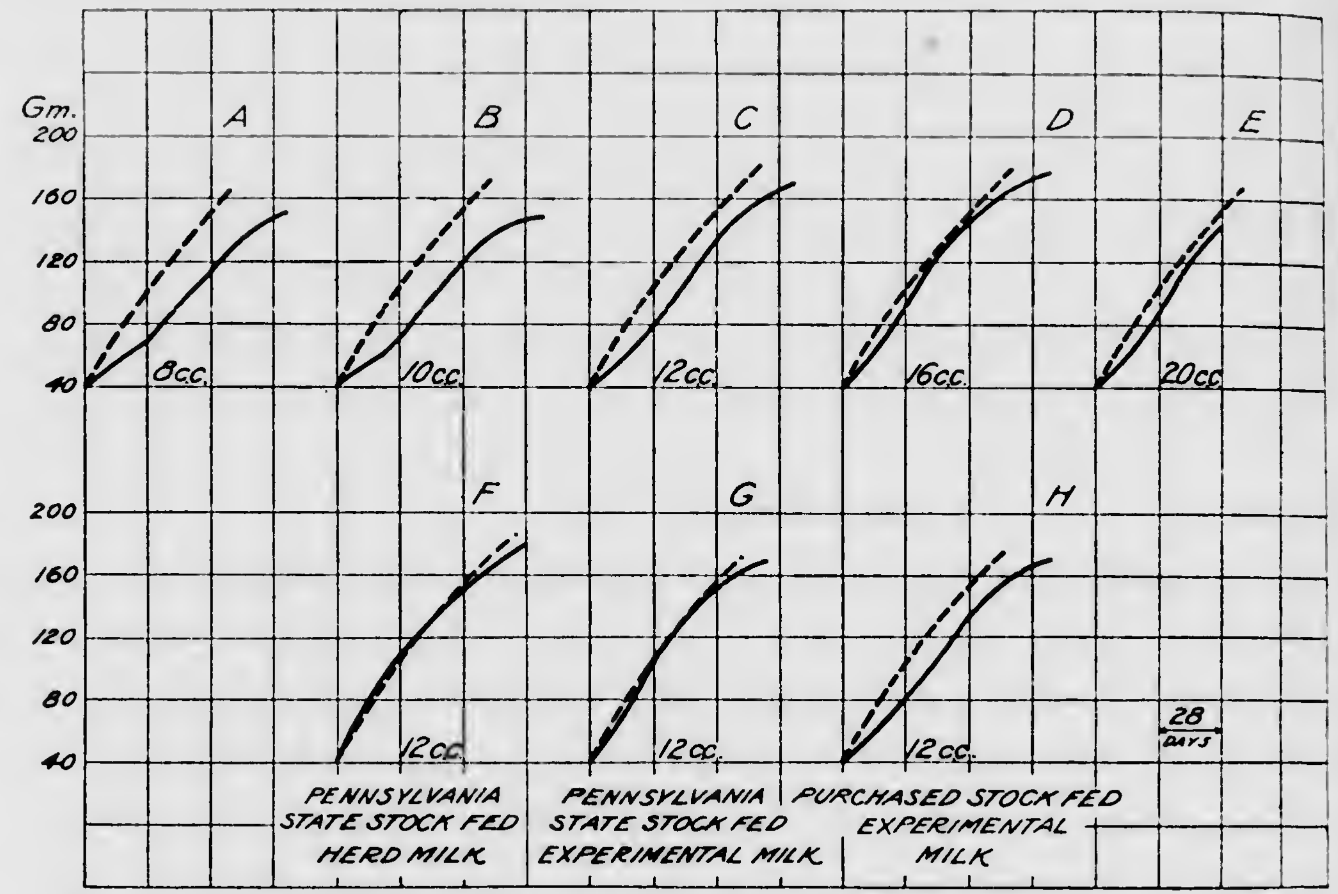

FIG. 1.—Growth curves. Broken lines represent normal growth of rats. Unbroken lines represent average growth of groups of rats fed different amounts of the experimental milk (excepting curve *F*, as indicated) as a supplement to a basal ration

The groups of rats allowed 16 c. c. (curve D) and 20 c. c. (curve E) of milk were so nearly identical that the experiment with the latter was discontinued after 56 days in order to save the milk for further feeding. Had the rats in these groups been of the Pennsylvania State stock their growth curve would no doubt have been above the Donaldson normal curve which is used in presenting all data from this laboratory. Furthermore, had the Pennsylvania State stock been used in the groups fed 8 and 10 c. c. of milk, the growth curves would have approached more closely to the normal.

The authors have for some time been conducting experiments to determine the possible synthesis of vitamin B by bacterial fermentations in the rumen of the digestive tract, as mentioned in an earlier paper (*2*). Although these experiments are not completed, it may be said at this writing (April, 1927) that some very positive evidence has been obtained.

SUMMARY

This investigation was conducted to determine the vitamin B potency of milk from three cows that were fed for over two years, throughout their growth period, on an experimental ration that was decidedly deficient in vitamin B growth factor. The milk was fed to rats as a supplement to a vitamin-B-free basal ration on levels of 8, 10, 12, 16, and 20 c. c. per rat per day. The vitamin B potency of the milk was found equal to that of herd milk from cows receiving a good winter ration. It is concluded that vitamin B in milk is not dependent upon the presence of this vitamin in the ration of the cow. It would appear that cattle, and possibly all ruminants, differ from other animals in their ability to grow to maturity, to produce normal offspring, and to maintain vitamin B in their milk when forced to subsist on rations deficient in vitamin B. If this is true, it would appear that ruminants possess the power to synthesize vitamin B. Experiments are in progress to determine whether this synthesis is due to microorganisms normally present in the rumen.

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A STUDY OF ARSENICAL RESIDUES ON APPLES IN PENNSYLVANIA WITH RESPECT TO EFFICIENT SPRAYING PRACTICES[1]

By G. F. MACLEOD, D. E. HALEY and R. H. SUDDS, *The Pennsylvania State College, State College, Penna.*

ABSTRACT

Insect species attacking apples in Pennsylvania necessitated six arsenical spray applications. The omission of one or more of these treatments resulted in increased injuries to the fruits. Satisfactory protection was obtained during 1926 without excessive arsenic occurring on the apples at harvest time. Rains in excess of normal tended to reduce the amount of spray deposit. Visibility of materials was no indication of the relative amounts of residual arsenic.

INTRODUCTION

Apples of the southern and southeastern counties of Pennsylvania are subject to attacks of several insects which ordinarily feed extensively during the middle and late summer months. Codling moth outbreaks occur late in August in some years. *Eulia velutiana* Walker, the red-banded leaf-roller; *Archips rosaceana* Harris, the oblique banded leaf-roller; and *Amorbia humerosana* Clemens, are late feeders of importance, some individuals appearing in October or during November.

The damage incurred from these insects is often a source of annual losses to fruit growers, and late summer sprays containing lead arsenate are sometimes necessary for the complete protection of apple fruits from insect injury. As a result, a rather heavy coating of material sometimes remains on the fruits. A few shipments of apples have been rejected or seized, both at home and abroad because of excessive spray residues. The financial losses and embarrassment sustained by growers on account of these procedures, together with the absence of local information concerning arsenical deposits led to a study of these conditions by the Department of Entomology Extension of the Pennsylvania State College. The analyses of residues were made by the Department of Agricultural and Biological Chemistry.

OBJECT OF THE STUDY

The object of this study was to ascertain quantitatively the amount of residual arsenic present on apples which had received the minimum

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[1] Approved by the Director of the Experiment Station as Technical Paper No. 434.

quantity of spray material required for the adequate protection of the fruits. This work comprised two phases,—(1) the determination of what constituted the minimum amount of arsenical spraying for complete protection and (2) the determination of arsenic in spray deposits.

Arsenical Spraying Practices During 1926

The spraying schedule as outlined in Table 1 includes the dates of applications which were advised in 1926.

Discussion

From Table 1 it will be observed that six applications of arsenical sprays were used in these sections. No arsenical was used in the delayed dormant period where oil sprays were applied. In either instance, however, the fruits received four poison sprays from the time of the petal fall treatment to the end of the spraying season.

The advisability of using arsenicals at the periods stated and in the amounts indicated is evident from the nature of the problems involved. Tent caterpillars, bud moths and leaf-rollers were unusually numerous during 1926, and a reduction in the numbers of the first generation assists greatly in preventing late summer injuries to the fruit.

Codling moth adults did not emerge in these counties at the time of the petal fall application, but ten to fifteen days later they appeared in large numbers. Coincident with this emergence, the plum curculio gave promise of unusual damages to fruits. These two factors were responsible for an emergency spray warning noted in Table 1. The usual cluster apple and summer applications were required by the presence of apple scab, together with the continued activities of the plum curculio, our common leaf-rollers, and the codling moth. The summer application was made from the middle to the last of July. Only two of the applications advised contained a spreader.

Effectiveness of Spray Applications in Commercial Orchards

The percentages of clean fruits recorded in Table 2 are only of apples which were not injured by insects ordinarily controlled by lead arsenate. As far as possible, Stayman apples were selected from vigorous, well cared-for trees in the prime of bearing condition. Orchard 16 received an application of sulfur-lead arsenate dust at the time of the last treatment. This represented an effort on the part of the grower to avoid excessive spray residues, and it is interesting to note that there was no visible residue on these apples, (see Table 3, sample No. 11). The degree of protection in this orchard was considerably less than that obtained where a liquid application was made. Orchard 17 did not

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Table 1.—Arsenical Spray Applications Advised in 1926

Period	Materials	Insects present	Dates of application by counties							
			Berks	Bucks	Chester	Delaware	Lebanon	Lehigh	Montgomery	Philadelphia
Delayed Dormant	Lime sulfur diluted to 1.03 Sp. gr. Lead arsenate 3 lbs. per 100 gals. Nicotine 1 pint per 100 gals.	Apple aphids Early worms Scale Tent caterpillars	April 21	April 22	April 19	April 19	April 21	April 23	April 19	April 19
Pre-Blossom	Lime sulfur diluted to 1.008 Sp. gr. Lead arsenate 3 lbs. per 100 gals. Nicotine 1 pint per 100 gals.	Apple scab Bud moths Leaf-rollers Apple aphids	May 6	May 5	May 4	May 3	May 6	May 7	May 4	May 3
Petal Fall or Calyx	Same as pre-blossom	Apple aphids Red bugs Codling moth Curuclio Apple scab	May 17	May 17	May 16	May 14	May 17	May 19	May 16	May 14
Emergency	Lime sulfur diluted to 1.008 Sp. gr. Lead arsenate 3 lbs. per 100 gals.	Apple scab Curculio Codling moth	May 27	June 1	May 27	May 27	May 28	June 8	May 27	May 28
Cluster Apple	Spreader Same as emergency	Apple scab Curculio Codling moth Leaf-rollers	June 16	June 8	June 8	June 8	June 15	June 17	June 8	June 8
Summer	Lime sulfur and Lead arsenate as in emergency	Codling moth Leaf-rollers	July 30	July 18	July 16	July 16	July 17	July 19	July 16	July 16

receive the emergency application. As a result, in this orchard there was an increased amount of curculio injuries. Orchard 18 was used to determine the intensity of insect injuries. The figures, Table 2, indicate the efficiency of the advised spraying practices.

TABLE 2.—RESULTS OF COMMERCIAL SPRAYING FOR FRUIT PROTECTION
Classification of Injuries†

Orchard No.	Treatment*	Plum Curculio Per cent	Fruit Worms Per cent	Codling Moth Per cent	Injured Fruit Per cent	Clean Fruit Per cent
1	As advised	1	0	0	1	99
2	As advised	0	2	2	4	96
3	Sprayed Personally	5	0	2	7	93
4	As advised	2	6	0	8	92
5	As advised	6	4	2	12	88
6	As advised	7	5	3	15	85
7	As advised	11	4	2	17	83
8	As advised	13	4	0	17	83
9	As advised	8	6	4	18	82
10	As advised	10	5	3	18	82
11	As advised	8	7	5	20	80
12	As advised	14	0	6	20	80
13	As advised	15	1	4	20	80
14	As advised	10	2	12	24	76
15	As advised	16	5	5	26	74
16	Last application a dust	14	4	11	29	71
17	Emergency application not used	15	12	6	33	67
18	Three applications at random	20	13	19	52	48

*"As advised" means that the orchard was treated according to the recommendations of the spraying service.

†The classification "Fruit Worms" is used to include all injuries caused by species such as green fruit worms, bud moths, and leaf-rollers.

The data expressed in Table 2 were obtained from 18 different orchards located in six different counties of southeastern Pennsylvania. In general, the results indicate that the number of arsenical treatments suggested were sufficient to protect the fruit. It is also evident that any diminution in the numbers of treatments could not have been expected to afford adequate fruit protection.

ARSENIC CONTENT OF THE SPRAY RESIDUES

METHODS OF SAMPLING

Samples consisting of 24 apples each, were selected in the orchards represented in Table 2 and in other plantings. A total of 33 orchards in 17 counties contributed samples for analyses. In picking the fruits those which had the heaviest spray deposit were secured. A square of heavy paper was placed around each apple and the fruit carefully removed from the branch. Each sample was packed in a cardboard carton and sent to the laboratory. All fruits were collected from the lower branches, where the maximum amounts of visible residue were found on the fruit before it was handled. A second collection was made from the same orchards, taking fruits from containers ready for market.

METHODS OF ANALYSIS

The different samples used were weighed before treatment for the removal of arsenic, and the AS_2O_3 calculated per pound of fruit.

The modified Gutzeit method[2] was used and gave satisfactory results. In removing the total quantity of arsenic from the surface of the apples it was found that a 7 per cent ammonium hydroxide solution gave approximately as satisfactory results as were obtained by the use of a mixture of hot concentrated nitric and sulfuric acid. In either case, care was taken to remove any residue from the stem, cavity and calyx of each apple, and the surface was thoroughly washed with water. The solution obtained was then made up to volume and an aliquot was taken for analysis.

All determinations were made under the same conditions as to length of time, temperature, acidity, amount and surface area of the zinc employed. The results obtained are given in Table 3.

The amount of rain fall is known[3] to have a direct influence on the amount of arsenic found in spray residues and should be considered in connection with the analysis. The samples analyzed were exposed to rains which are indicated in the graph, Table 4. During the months of April, May, June and July there was a total deficiency in rainfall of 4.73 inches, but there was an excess of 5.12 inches through August, September and October.[4] Since but three of the samples analyzed were sprayed after July 31, the majority of the fruits were subjected to rains in excess of the normal precipitation.

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[2] Scott, W. W., 1917, Standard Methods of Chemical Analysis. P. 40. New York City.

[3] O'Kane, W. C., Hadley, C. H., and Osgood, W. A., Bulletin No. 183, N. H. Agricultural Experiment Station.

[4] U. S. Weather Bureau, Philadelphia, Pa., 1926.

Table 3.—Results of Arsenical Analyses

Sample No.	Location County	No. Ars. sprays	Date last application	Amount spray residue	Date collected	Amount AS_2O_3 per lb. apples Grains
1	Montgomery	3	July 16	Light to medium	Oct. 6	0.0019
2	Montgomery	3†	July 16	Heavy	Oct. 6	0.0026
3	Bucks	2	June 1	Light	Oct. 7	0.0003
4	Bucks	3	July 18	Light	Oct. 7	0.0010
5	Lehigh	2 & 1 dust*	July 19	Light	Oct. 8	0.0004
6	Lehigh	3	July 19	Heavy	Oct. 8	0.0013
7	Philadelphia	3	July 16	Heavy	Oct. 12	0.0018
8	Philadelphia	3	July 16	Heavy	Oct. 12	0.0014
9	Berks	3	July 30	Heavy	Oct. 14	0.0004
10	Berks	3	July 30	Heavy	Oct. 14	0.0004
11	Delaware	2 & 1 dust*	Aug. 6	Very light	Oct. 19	Trace
12	Delaware	2 & 1 dust*	July 15	Medium	Oct. 19	0.0004
13	Lebanon	3	July 17	Light	Nov. 4	0.0004
14	Lebanon	3	July 17	Light	Nov. 4	0.0020
15	Adams	4 dusts	June 30	None	Oct. 8	Trace
16	Adams	3 sprays	July 1	Light	Oct. 8	0.0002
17	Adams	4	July 23	Heavy	Oct. 8	0.0009
18	Franklin	2	July 2	Medium	Oct. 9	Trace
19	Franklin	4	July 20	Heavy	Oct. 9	0.0007
20	Franklin	4	July 26	Heavy	Oct. 9	0.0013
21	Franklin	4 & 1 dust*	Aug. 1	Light	Oct. 9	0.0005
22	Lycoming	3	July 31	Heavy	Oct. 12	0.0007
23	Lycoming	3	July 31	Medium	Oct. 12	0.0004
24	Lycoming	3	July 31	Heavy	Oct. 12	0.0005
25	Lycoming	4	Aug. 2	Very heavy	Oct. 12	0.0059
26	Lycoming	4	Aug. 2	Very heavy	Oct. 12	0.0057
27	Lycoming	4	Aug. 2	Very heavy	Oct. 12	0.0059
28	Lycoming	4	Aug. 2	Very heavy	Oct. 12	0.0041
29	Carbon	3	July 1	Very light	Oct. 14	0.0008
30	Carbon	3	June 11	Very light	Oct. 14	Trace
31	Carbon	3	July 7	Very light	Oct. 14	0.0005
32	Schuylkill	3	July 10	None	Oct. 15	0.0005
33	Schuylkill	4	July 15	Light	Oct. 15	0.0010
34	Mifflin	4	July 26	Light	Oct. 22	0.0005
35	Mifflin	4	July 26	Medium	Oct. 22	0.0007
36	Mifflin	4	July 26	Heavy	Oct. 22	0.0017
37	Juniata	3	July 3	Medium	Oct. 22	0.0007
38	Lawrence	3	Aug. 5	Medium	Oct. 23	0.0012
39	Lawrence	3	Aug. 5	Medium	Oct. 23	0.0010
40	Washington	3†	Aug. 1	Light	Oct. 23	0.0004
41	Washington	3	Aug. 1	Light to Medium	Oct. 23	0.0035
42	Fayette	3	Aug. 1	Medium	Oct. 22	0.0019

*Last application.

†Colloidal lead arsenate.

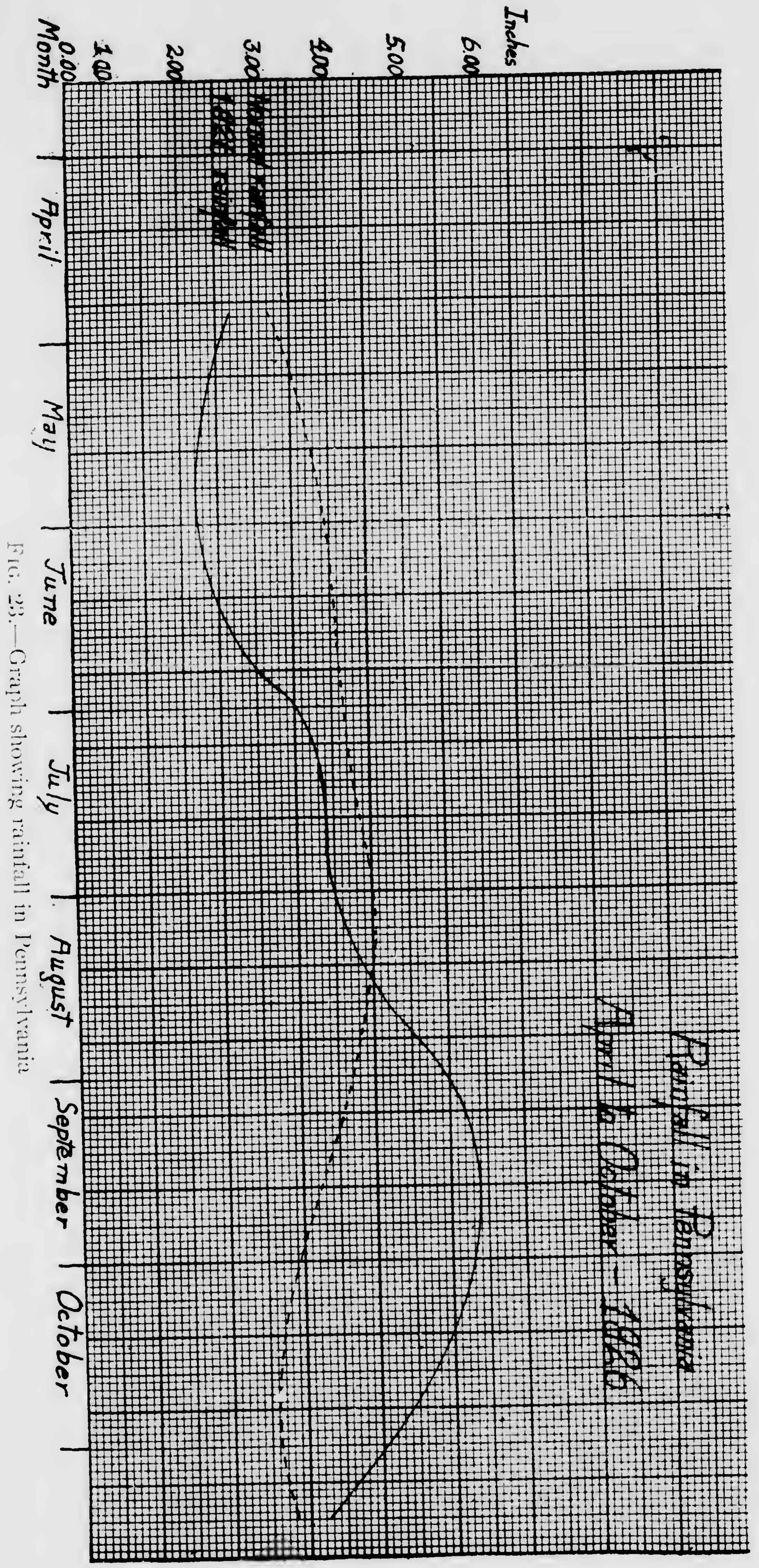

Fig. 23.—Graph showing rainfall in Pennsylvania

The analytical findings seem to justify certain conclusions. Arsenic, with but one exception, was found on all samples tested. In no case was there found an amount of arsenic equivalent to 1/100 of a grain per pound of apples, the stated British tolerance. These results, however, were all made previous to any handling of the fruit. When the apples were presented for public consumption the amount of arsenic found was reduced to a trace, (see Table 5).

It was found that the relative quantity of visible spray residue did not serve as an index of the *quantity* of arsenic present. As a matter of fact, as much arsenic was found on apples having no visible spray residue, as on those having considerable residual material on their surface.

Different samples obtained from the same orchard and receiving the same treatment showed a uniform quantity of arsenic present.

TABLE 5.—AMOUNTS OF ARSENIC ON FRUITS BEFORE AND AFTER HANDLING

Sample No.	Amount of visible spray residue		Amount of arsenic (AS_2O_3)	
	Before handling	After handling	Before handling	After handling
			Grains per pound	
1	Light	None	0.0019	Trace
2	Heavy	Very light	0.0026	Trace
3	Light	None	0.0003	Trace
4	Light	None	0.0010	Trace
5	Heavy	None	0.0004	Trace
6	Heavy	None	0.0004	Trace
7	Light	None	0.0004	Trace
8	Light	None	0.0020	Trace
9	Light	None	Trace	Trace
10	Light	None	0.0004	Trace

SUMMARY

Under conditions prevailing in Pennsylvania during 1926, it was found commercially feasible to produce marketable apples without objectionable spray residues. Sprays were applied with but little, if any, deviation from the usual procedure and no supplementary preparation of the fruit was necessary.

Visibility of spray deposits on these apples was no indication of the relative quantities of the poison.

The authors recognize that there were imperfections of methods in sampling and analysis, unusual climatological conditions, and that the study was of comparatively short duration. The results are presented, however, as a regional contribution to a subject of national interest.

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VITAMIN STUDIES.

XVI. VITAMIN A IN EVAPORATED MILKS MADE BY VACUUM AND AERATION METHODS.*

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(Received for publication, August 8, 1927.)

The first paper of this series (1) described the results obtained in an attempt to determine whether or not vitamin B is destroyed when cow's milk is subjected to commercial methods of evaporation.

The study described in the present paper, which is the second in the series, was undertaken with the view of answering a similar question with regard to vitamin A. We pointed out in the previous paper, to which we have just referred, that:

> "In the past practically all of the data available, relative to the vitamin content of evaporated milks, have been obtained on samples the past history of which has not been known. Furthermore, few attempts have been made to compare the evaporated milks with the raw milks from which they were made. As a result few authoritative data have been obtained concerning the influence of commercial evaporation processes on the stability of the various vitamins.
>
> The present studies were undertaken with the view of preparing evaporated milks under conditions approximating, as closely as possible, the conditions used in commercial practice and comparing these products with the original milk from which they were made, to eliminate the effect of any fluctuation in vitamin content that may take place in raw milk obtained from various sources and at different seasons of the year."

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* Published with the permission of the Director of the Agricultural Experiment Station as paper No. 438.

Evaporated Milks.

For details of the manufacturing processes which were carried out on a semicommercial scale in our college creamery, the reader is referred to our former paper (1). For the sake of clearness, however, it is necessary to say that two types of evaporated milks were studied: (1) evaporated milk made by the vacuum process, and (2) evaporated milk made by the aeration process. The former was representative of most commercial evaporated milks sold on the market, having been made in the copper vacuum pan in the absence of air. The aerated milk was representative of a type which is of less commercial importance in the evaporated milk industry but was studied because of the scientific interest associated with vitamin destruction by oxidative processes. Portions of both of these types of milks were sterilized by the fractional sterilization method. These sterilized samples were studied with the view of determining the effect of sterilization as it is practiced in all condenseries where evaporated milks are made.

The certified milk, from which the evaporated milks were made, was obtained fresh from the barn every morning. The evaporated milks, which were made at 4 or 5 week intervals, were analyzed and diluted to the concentration of the original raw milk before they were fed. In other words, all evaporated milks were compared directly with equal volumes of the certified milk from which they were made, the latter being used as the standard.

In most of the work reported by other workers it has not been possible to say with certainty whether or not vitamin A destruction occurred during commercial evaporation, owing to a lack of knowledge regarding the vitamin content of the original raw milk, which is known to fluctuate in vitamin potency depending on the vitamin content of the dairy ration (2).

As a result, five types of milk were fed: (1) raw milk from the certified herd (R. M.), (2) evaporated milk made by the vacuum process (V. E.), (3) evaporated milk made by the vacuum process and sterilized (V. E. S.), (4) evaporated milk made by the aeration process (A. E.), and (5) evaporated milk made by the aeration process and sterilized (A. E. S.).

During the year 1925 a study was made using the old prophylactic method of feeding. The rats, which numbered 150, were fed separately in individual cages and on screens. The rats were weighed weekly and food intake records were kept for each individual.

The ration, which was not irradiated, consisted, in parts per 100, of casein 18, agar 2, McCollum's salt mixture[1] (No. 185) 3, and dextrin 77. To insure an adequate and constant supply of vitamin B, 0.5 gm. of Fleischmann's special dried yeast was fed (daily) separate from the ration. The experimental milks were fed from the beginning of the experiment in special feeding dishes and no difficulty was experienced in obtaining complete consumption of the milk. No attempt was made at that time to provide for a supply of the antirachitic factor. Chart 1 summarizes the results obtained by the prophylactic method. Each curve represents the averages of ten or more animals and the abbreviations refer to the different types of experimental milks which were described in a previous paragraph.

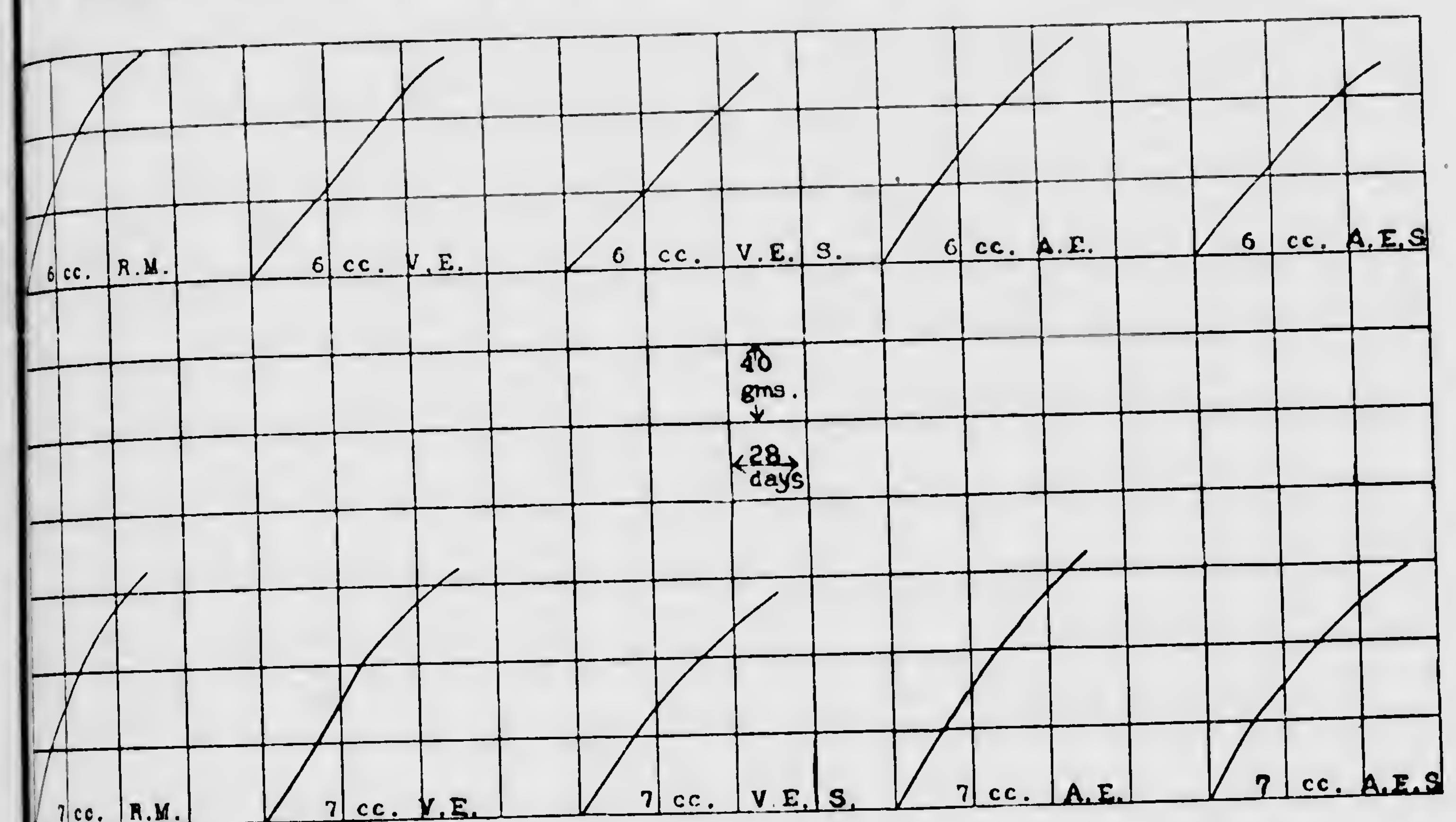

Chart 1. Vitamin A in raw and evaporated milks as measured by the prophylactic method of feeding.

Previous work, in which the prophylactic method of feeding (2) was used, showed that approximately 10 cc. of raw milk are necessary to furnish sufficient vitamin A for normal growth in rats. Later work, in which the prophylactic method was used, but with the ration irradiated, gave us results which showed that considerably less milk was required for fairly satisfactory growth. This is in agreement with the work of Chick and Roscoe (3) who noted that a smaller amount of milk was required to furnish sufficient vitamin A when the curative method was used and when the ration contained irradiated vegetable oil. These workers, using the normal rate of growth as their standard, endeavored to find the amount of milk necessary to produce an average gain of 10 to 13 gm. per week for a curative period of 4 weeks following the prefeeding period on the vitamin A-free diet.

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[1] McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, xxxiii, 63.

Sherman and Munsell (4) have used similar technique, with the exception that they have felt that it is not necessary to take special precautions regarding the furnishing of the antirachitic factor by irradiation. The Sherman-Munsell standard also differs from that of Chick and Roscoe in that Sherman and Munsell make no attempt to obtain the normal growth rate during the curative period. Instead, they have sought to find the minimum quantity of food which will furnish sufficient vitamin A to prevent loss of body weight and produce a slight but steady growth. They have suggested that this technique be standardized by finding that amount of food which will produce an average weekly increase in body weight of 3 gm. for a period of 8 weeks following the depletion of the body reserves.

Javillier and coworkers (5) have suggested a similar but higher standard. Using the curative method also, they attempt to feed that amount of food which will produce, for 30 days, an increase in body weight of about 30 per cent and an angle of growth of 30 degrees when days and gm. are given the same linear value on the chart. This standard is what might be termed, equivalent to about one-half of normal growth.

EXPERIMENTAL.

In 1926 we repeated our experiments but modified the technique by using the curative method of feeding and the basal ration was irradiated in shallow pans with a Cooper Hewitt Uviarc lamp at a distance of 24 inches for 15 minutes, the ration being stirred at intervals of 5 minutes during the irradiation. Previous work in our laboratory (6) has shown that dextrin prepared from commercial corn-starch can be activated to furnish sufficient antirachitic factor to eliminate the possibility of insufficient calcium and phosphorus utilization. We have not yet made a study of this phase of the problem but we feel that the activation of our dextrin is probably due to the presence of sterols in the impure commercial starch from which the dextrin was made.

A total of 177 rats was used in this portion of the work, all animals being fed on screens and in individual cages. The basal ration was of the same composition as that described in the discussion of our 1925 experiments and Fleischmann's[2] special yeast was fed daily in individual dishes separate from the ration.

All rats were placed on experiment at 21 days of age at a weight of about 40 gm. Animals which weighed less than 37 gm. or more than 42 gm. were not used. These precautions were taken with the hope that we might obtain more uniform responses to our experimental procedure. Our stock ration (7) is of constant composition and we should expect that vitamin storage would not vary appreciably in our experimental rats.

This expectation has been borne out by our experience during the past year. A large proportion of our rats, when placed on the vitamin A-free basal ration will have depleted their vitamin A body reserves in 35 to 40 days. By far the greatest number will have reached this point on the 37th day of the prefeeding period, at which time the curative food is introduced. Since the prefeeding period requires 5 weeks for vitamin A depletion, we have arbitrarily chosen the same period (5 weeks) for the curative feeding, the entire experiment occupying 10 weeks.

Preliminary feeding trials with various amounts of raw milk led us to believe that 1 cc. would produce an average weekly gain approximating the Sherman-Munsell standard of 3 gm. per week. Likewise, we had evidence that 1.5 to 2.5 cc. should be sufficient to bring about a rate of growth approximately equal to the Javillier standard of 6 gm. per week. With these facts in mind the various experimental evaporated milks were fed at 1, 1.5, and 3 cc. levels. In no case were there less than six rats in an experimental group and in several groups the number of experimental animals exceeded this figure, the highest being thirty-one animals in the group receiving 2 cc. of raw milk. The rats were not all

[2] The writers wish to acknowledge their indebtedness to Dr. Robert E. Lee of the Fleischmann Yeast Company who furnished the yeast used in all of our feeding work.

placed on experiment at one time, a number being added to each experimental group from time to time throughout the year. This was done to eliminate seasonal variation influences in case the milk should vary slightly from season to season during the experiment. Males and females were distributed evenly in all groups and, so far as possible, litter mates were distributed in the same manner.

TABLE I.

Average Weekly Growth of Rats Receiving Raw and Evaporated Milk.

Milk fed.	Number of rats in group.	Average weight of rats.		Total gain.	Average gain per wk.	Growth.	Growth injury.
		At start.	After 5 wks.				
cc.		*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1.0 R. M.	13	63.5	78.3	14.8	2.96	100.00	0.00
1.0 V. E.	8	54.2	63.8	9.6	1.92	64.86	35.14
1.0 V. E. S.	6	53.0	61.3	8.3	1.66	56.08	43.92
1.0 A. E.	6	55.5	61.1	5.6	1.12	37.83	62.17
1.0 A. E. S.	6	52.0	57.1	5.1	1.02	34.45	65.55
1.5 R. M.	14	56.9	83.9	27.0	5.40	100.00	00.00
1.5 V. E.	8	52.5	70.6	18.1	3.62	67.03	32.97
1.5 V. E. S.	9	62.2	80.2	18.0	3.60	66.66	33.34
1.5 A. E.	11	64.2	82.0	17.8	3.56	65.92	34.08
1.5 A. E. S.	10	60.4	77.8	17.4	3.48	64.44	35.56
2.0 R. M.	31	64.2	91.5	27.3	5.46		
2.5 R. M.	10	63.1	94.2	31.2	6.22		
3.0 R.M.	10	63.9	107.3	43.4	8.68	100.00	00.00
3.0 V. E.	9	62.7	99.7	37.0	7.40	85.25	14.75
3.0 V. E. S.	9	63.8	100.8	37.0	7.40	85.25	14.75
3.0 A. E.	7	67.7	104.2	36.5	7.30	84.10	15.90
3.0 A. E. S.	10	74.2	108.0	33.8	6.76	77.88	22.12

When our rats have subsisted on the vitamin A-free basal ration for about 21 days, growth is retarded. By the 28th day, as a rule, growth has ceased and the curve remains flat until the 35th to 37th day when an abrupt fall occurs. At this point the animal will lose from 3 to 6 gm. in a day. It is at this point that we feed the curative food containing vitamin A.

Table I summarizes the data obtained by the curative method. A study of Table I shows that better growth was obtained in every case where raw milk was fed than was obtained after the milk had been evaporated. It will be noted that the average weekly rate of growth on 1 cc. of raw milk (R.M.) was practically equal to the Sherman-Munsell standard. When the milk is evaporated by the vacuum process (V.E.), diluted to its original concentration, and fed at the 1 cc. level in direct comparison with the raw milk, the average weekly growth is 64.86 per cent of that obtained on 1 cc. of raw milk. After sterilization (V.E.S.) by the fractional method described in the first paper of this series, growth is but 56.08 per cent of that obtained with an equal amount of raw milk.

When milk was evaporated by the aeration method (A.E.) growth is but 37.83 per cent and after sterilization (A.E.S.) growth is but 34.45 per cent of that obtained on 1 cc. of raw milk. We are unable to explain the slight difference between the average weekly gains obtained with 1.5 and 2.0 cc. of raw milk (R.M.) in spite of the fact that the groups contained fourteen and thirty-one animals, respectively. At the 2.5 cc. level (R.M.) the average gain per week is practically identical with the Javillier standard of 6 gm. per week.

When the experimental milks were fed at the 1.5 cc. level the same general relationship is noted except that the average weekly growth is better. When the 1.5 cc. (R.M.) growth average is used as the standard the vacuum evaporated (V.E.) group is 67.03 per cent while sterilization (V.E.S.) has reduced the growth slightly, although it is doubtful if such slight differences are significant.

At the 3 cc. feeding level the relation of one group to another is about the same as the ones discussed above with the exception that growth in all groups was much better, which should be expected. It is significant to note that the effect of temperature and aeration becomes less marked at the highest level of feeding while the effects are quite marked in those groups receiving the least amount of milk. It is probable that slight quantitative differences in the vitamin content of foods cannot be determined with the same accuracy by using normal rates of growth as can be obtained with standards of the Sherman-Munsell type. On the other hand, we have observed a much greater proportion of respiratory troubles and infections of various types when animals are allowed to grow but 3 gm. per week than we have observed in animals growing at the rate of 6 gm. per week. This is in general agreement with the findings of Macy and coworkers (8), although they use normal growth (12 to 14 gm. per week) as their standard. We have not been able to obtain the growth response on raw cow's milk obtained by the last mentioned investigators (9), who obtain average weekly growth, by the curative technique, of 12 to 13 gm. per week on 3 cc. of cow's milk, while we obtain weekly gains of 8 to 9 gm. on the same amount of milk. It is possible that the environment and diet of the cows may account for this difference. The herd from which our milk was obtained received the same ration the year round, had no access to pasture, and received but a limited and variable amount of green feed during the summer months in the form of green hay.

We have not been able to depend on the onset of ophthalmic symptoms as an index of vitamin A depletion for the reason that many of our animals show no signs of this condition at the time that the body weight becomes stationary, following the prefeeding period. In fact, an examination of our records shows a much smaller percentage of animals afflicted with xerophthalmia than is reported by many workers. In this study not more than 30 per cent of our animals showed evidences of the disease. In the past, when we irradiated the animals directly, we obtained xerophthalmia in about 50 per cent of the cases on record. As soon as we abandoned direct irradiation in favor of food irradiation, the number of cases fell to about one-half of the former figure.

Postmortem records were kept of all animals and particular attention was paid to ear, lung, and tongue infections. We have been able to find but one or two cases of tongue infections as described by Macy and coworkers. In those groups which grew at a rate of 3 gm. or less per week we observed a greater number of cases of ear and lung infection than in the groups which grew at 6 gm. or more per week. In general, the infections were less severe at the higher growth levels than at the lower levels, although there were a few exceptions to this generalization.

We have expressed, in Table I, the percentage of loss in weight or failure to grow, using the raw milk groups as standards. We make no attempt at this time to take a definite stand for any of the growth standards discussed. We have the feeling that normal growth standards are not capable of detecting small differences in the vitamin content of foods while we are also somewhat disturbed when we apply the Sherman-Munsell standard for the reason that we are in doubt regarding the influence of lowered resistance and infection on the results. While we are not yet ready to advocate it, we are inclined to favor the "half normal" standard of Javillier, since health is fairly good at that rate of growth and biological response to small food additions seems to us to be fairly well marked.

It would appear that vacuum and aeration methods of evaporation possess an appreciable destructive effect on the vitamin A content of milk. We feel that this destructive effect cannot be due to the effect of heat on the mineral content of the milk for the reason that McCollum's Salt Mixture 185 was fed throughout the experiment. It would appear, therefore, that the loss in nutritive value must be due to destruction of vitamin A. While it is impossible to draw accurate conclusions regarding the amount of vitamin A destroyed it would appear that it may vary from 10 to 30 per cent, depending upon the treatment the milk has received and upon the growth standards used in making the interpretations.

SUMMARY.

Experiments are described in which rats were fed a vitamin A-free, irradiated basal ration, the curative technique being used. Evaporated milks made by vacuum and aeration methods were fed, at various levels, in direct comparison with the raw milk from which they were made. It is concluded that evaporated milks made by the vacuum process have lost some of their nutritive value during the manufacturing process and that this loss, which does not appear to be unduly great, is probably due to destruction of vitamin A. It would appear also that aeration and sterilization increase this destructive effect to some extent. Quantitative interpretations of the destruction of vitamin A are discussed.

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A BIOLOGICAL MEASUREMENT OF AVAILABLE SOIL POTASSIUM[1]

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Various methods have been used in the past to measure the availability of the more important soil constituents. These methods have involved extraction of the soil with water, carbonated water, salt solutions, and dilute organic and inorganic acids. Mineral acids of relatively high concentrations have also been used. It is now recognized, however, that although certain of these solvents may serve to determine the availability of a single soil constituent, the same will not necessarily hold true in the case of other soil constituents.

At the present time, studies are being made of methods other than those involving extraction with solvents. Among these, that of measuring the availability by means of plants grown on the soils tested, and followed by determinations of the soil constituents absorbed, has been used to a certain extent.

A considerable amount of investigational work has been conducted on the availability of soil potassium, on account of the fact that the ordinary agricultural soils contain sufficient quantities of this element to suffice for the growth of plants for an indefinite period, providing it is rendered soluble. Therefore, a study of the factors influencing the solubility of soil potassium should be of interest both from a practical and scientific point of view.

From the standpoint of plant nutrition, any information pertaining to the quantity of potassium available in soil at any one time, or during a growing season, or as to the form in which it occurs, is of considerable importance, and for these reasons, in part, the following investigation was undertaken.

OBJECT OF THE EXPERIMENT

The object of this experiment was to measure the availability of potassium in two soils of known history, by means of plants grown in direct contact with them, under controlled conditions, and to determine the quantity of this element absorbed. In order to do this a modification of the method of Neubauer and Schneider (4) was employed. The original method is described in detail by Ames and Gerdel (2).

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[1] Published by permission of the Director of the Agricultural Experiment Station as scientific paper No. 431.

EXPERIMENTAL

Description of materials used

The soils used in this study were taken from plots 1 and 4, tier II, of the general fertilizer experiments of the Pennsylvania Experiment Station. These plots have been cultivated and cropped continuously since 1881. Plot 1 received no fertilizer during this time but plot 4 was treated with 200 pounds of muriate of potash biennially, and when sampled had received 18 such applications, the latest of these dressings being applied 14 months prior to sampling. These soils have been described by Frear and Erb (3), who used them for their work on a study of the condition of soil potassium as determined by means of solvents.

The medium for plant growth used throughout this experiment was pure white sand which had been thoroughly washed with tap water and finally with distilled water.

TABLE 1

Composition of nutrient solution

MATERIAL	AMOUNT	VOLUME	APPLICATION
	gm.	*cc.*	*cc.*
$(NH_4)_2HPO_4$	14.4908	8000	10
$CaH_4(PO_4)_2 \cdot H_2O$	25.9389	8000	10
$Ca(NO_3)_2 \cdot H_2O$	68.5225	8000	10
$MgSO_4 \cdot 7\ H_2O$	99.7879	8000	10
K_2SO_4	9.1661	2500	10
$FeCl_3 \cdot 6\ H_2O$	0.1000	250	1

Since buckwheat absorbs potassium in relatively large quantities and grows well under greenhouse conditions, this plant was chosen as the crop to be grown, and in order to insure a good growth, only carefully selected and healthy seeds were planted.

Fifteen seeds were added to each of the jars, and after germination six or seven plants were allowed to develop and the others were removed. The jars contained 2,750 gm. of sand, alone, or with varying quantities of soil.

A complete nutrient solution was added to those jars not receiving a potash addition in the form of soil. Where soil was present, potash was omitted from the nutrient solution. The composition and rate of application of this solution are given in table 1.

Each jar was kept at approximately 14 per cent moisture content with distilled water throughout the experiment.

The amounts of treated and untreated soils added to the various jars and thoroughly mixed with the sand before moisture was added, were 12.5, 25.0, 37.5, and 50.0 gm.

The first application of the nutrients was made on July 12, one week after

TABLE 2

Additions to the quartz sand medium in which plants were grown

JAR NUMBERS	SUPPLEMENTAL TREATMENTS				
	Soil additions		Nutrient additions		
	Plot 1	Plot 4	Kind of solution	Quantity applied	Time of application
	gm.	*gm.*			
1- 2- 3- 4	.....	.....	Complete	Single	Weekly
5- 6- 7- 8	.....	.....	Complete	Single	Semiweekly
9–10	.....	.....	Complete	Double	Weekly
11–12–13–14	.....	.....	K-free	Single	Semiweekly
15–16–17–18	.....	12.5	K-free	Single	Semiweekly
19–20–21–22	.....	25.0	K-free	Single	Semiweekly
23–24–25–26	.....	37.5	K-free	Single	Semiweekly
27–28–29–30	.....	50.0	K-free	Single	Semiweekly
31–32–33–34	12.5	.....	K-free	Single	Semiweekly
35–36–37–38	25.0	.....	K-free	Single	Semiweekly
39–40–41–42	37.5	.....	K-free	Single	Semiweekly
43–44–45–46	50.0	.....	K-free	Single	Semiweekly

TABLE 3

Total yield of dry matter and quantity of potassium absorbed

JAR NUMBER	NUMBER OF PLANTS	WEIGHT OF DRY MATERIAL	WEIGHT OF K ABSORBED	JAR NUMBER	NUMBER OF PLANTS	WEIGHT OF DRY MATERIAL	WEIGHT OF K ABSORBED
		gm.	*mgm.*			*gm.*	*mgm.*
1	6	2.56	77.4	3	7	1.81	68.3
2	6	3.06	76.5	4	7	2.90	69.9
5	6	3.73	136.9	7	7	4.99	161.8
6	6	3.06	121.6	8	7	5.26	159.6
9	6	4.95	157.1	.....	.....	....	......
10	6	3.89	149.1	.....	.....	....	......
11	6	0.77	2.7	13	7	0.79	3.1
12	6	0.57	2.6	14	7	0.64	2.6
15	6	0.99	4.2	17	7	1.19	6.4
16	6	1.23	6.1	18	7	1.63	7.6
19	6	1.66	....	21	7	1.73	......
20	6	1.81	....	22	7	1.82	9.2
23	6	2.28	11.7	25	7	2.13	11.1
24	6	2.14	10.8	26	7	3.16	13.3
27	6	3.55	16.1	29	7	3.23	11.9
28	6	3.73	17.5	30	7	4.06	17.6
31	6	1.05	3.7	33	7	1.11	4.7
32	6	1.06	3.5	34	7	1.06	5.7
35	6	1.06	5.7	37	7	1.49	6.6
36	6	0.90	5.4	38	7	1.06	5.9
39	6	1.44	8.1	41	7	0.96	5.1
40	6	1.27	7.9	42	7	1.24	7.2
43	6	1.46	7.9	45	7	1.38	7.2
44	6	1.28	7.6	46	7	1.32	7.6

planting, followed by successive additions at weekly or semiweekly intervals according to the plan of the experiment.

The jars were weighed daily, care being taken to prevent undue evaporation, by white-washing the greenhouse roof and by keeping the floors well sprinkled with water.

TABLE 4

Availability of potassium in the soils of plot 4 (treated) and plot 1 (untreated)

JAR NUMBER	PLOT NUMBER	WEIGHT OF SOIL	NUMBER OF PLANTS	POTASSIUM REMOVED IN EXCESS OF CHECK	CONCENTRATION OF AVAILABLE K IN SOIL
		gm.		*mgm.*	*per cent*
15	4	12.5	6	1.4	0.0112
16	4	12.5	6	3.3	0.0264
17	4	12.5	7	3.4	0.0272
18	4	12.5	7	4.8	0.0384
19	4	25.0	6	.....	......
20	4	25.0	6	.....	......
21	4	25.0	7	.....	......
22	4	25.0	7	6.4	0.0256
23	4	37.5	6	8.9	0.0237
24	4	37.5	6	8.0	0.0213
25	4	37.5	7	8.3	0.0221
26	4	37.5	7	10.5	0.0280
27	4	50.0	6	13.3	0.0266
28	4	50.0	6	14.7	0.0294
29	4	50.0	7	9.1	0.0182
30	4	50.0	7	14.8	0.0296
31	1	12.5	6	0.9	0.0072
32	1	12.5	6	0.7	0.0056
33	1	12.5	7	1.9	0.0152
34	1	12.5	7	2.9	0.0232
35	1	25.0	6	2.9	0.0116
36	1	25.0	6	2.6	0.0104
37	1	25.0	7	3.8	0.0152
38	1	25.0	7	3.1	0.0124
39	1	37.5	6	5.3	0.0141
40	1	37.5	6	5.1	0.0136
41	1	37.5	7	2.3	0.0061
42	1	37.5	7	4.4	0.0117
43	1	50.0	6	5.1	0.0102
44	1	50.0	6	4.8	0.0096
45	1	50.0	7	4.4	0.0088
46	1	50.0	7	4.8	0.0096

The plants started to bloom on July 30. In order to insure more growth, these blooms were removed and preserved for the final harvest, which was on August 23. At that time the plants were cut close to the sand, placed in large envelopes, and transferred to a drying closet. After the plants were dried, they were ground and preserved for analysis.

At the time of harvesting, an attempt was made to recover the roots. Unfortunately, however, it was found impossible to remove them in their entirety. It was observed, however, that a rather excellent root development was obtained where the plants were grown in the presence of 50 gm. of treated soil, the roots extending to the bottom and throughout the mass of sand and soil, tending to hold it together.

All plants grown in the presence of soil showed pronounced symptoms of potash starvation. Where 50 gm. of the treated soil was used, however, these symptoms did not appear until a week to ten days before harvesting. It was unfortunate that larger quantities of soil were not used in all cases, but the main endeavor in this experiment was to make potassium the limiting factor of plant growth.

The method employed for the determination of potassium in the plants was the same as used by Ames and Boltz (p. 206).

In every case, the entire yield of plants of each jar was taken for individual analysis.

Presentation of data

Forty-six jars were used in the experiment. To some of these were added complete or K-free nutrients in single or double quantities and at intervals of every week or twice a week. To some of the jars were added varying quantities of treated and untreated soils. The supplemental treatments of the jars are given in table 2.

With one exception, two sets of duplicates were run on all individual treatments, one set having 6 plants and the other 7 plants per jar. The total yields of material obtained from each jar, together with the quantity of potassium absorbed are given in table 3.

Previous investigational work on the amount and condition of potassium in each of these two soils, as conducted by Frear and Erb (3) is given below:

AMOUNT OF POTASH	IN TERMS OF AIR-DRY FINE SOIL	
	Plot 1	Plot 4
	per cent *K_2O*	*per cent* *K_2O*
Total	3.8210	3.5430
Soluble in hot 1.115 sp. gr. HCl, 10 hours, 1:10	0.3687	0.4092
Soluble in warm 40°C., 0.2 *N* HCl, 5 hours, 1:10	0.0143	0.0301
Soluble in distilled water,		
Flask method, 5 hours, 40°C., 1:10	0.0032	0.0049
Flask method, highest results	0.0046	0.0075
Percolation method, 21°C., 1:10, 4 to 6 days	0.0045	0.0080
In water from clay washing	0.0038	0.0087
Soluble in carbonated water by percolation, 21°C., 1:10, 2 to 5 days	0.0076	0.0140
Soluble in 0.33 *N* NH_4Cl solution, 5 hours, 40°C., 1:10		
First extraction	0.0094	0.0187
Second extraction	0.0020	0.0021

The quantities of potassium absorbed by the six or seven plants of each set of duplicates of the soil treatments after subtracting the quantity found in the plants receiving the K-free nutrients, are given in table 4.

DISCUSSION OF RESULTS

The results obtained by this preliminary experiment, although not conclusive, show that the plants absorbed more potassium from the "treated" soil than from the "untreated." It is also shown that plants must attain a certain condition of growth before they serve as a good index for determining the condition of potassium in soils. In an experiment of this nature, potassium must necessarily be the limiting factor of growth, but better results would doubtless have been obtained if more soil had been used.

Because of the variation normally in evidence where but few plants are grown, there were slight variations in the quantities of potassium absorbed where the plants were grown under the same environmental conditions. No great difference was noted in the quantities absorbed by the six and the seven plants. Calculating the quantity of potassium absorbed by each set of plants, in excess of that absorbed by the check, and averaging the percentages obtained, it was found that the potassium available for plant growth in the treated soil amounted to 0.0252 per cent, and in the untreated soil, 0.0115 per cent. These are practically the same figures obtained by Frear and Erb (3) from their studies of these soils using 0.2 *N* HCl, namely 0.0250 and 0.0118 per cent of potassium, respectively.

SUMMARY

This preliminary experiment suggests a method of attack for determining the availability of potassium in soils. Although the results are meager and do not warrant any definite conclusions, nevertheless, there seems to be a close correlation between the quantities of potassium absorbed by buckwheat plants above the roots and those obtained by the use of 0.2 *N* HCl as a solvent.

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A STUDY OF THE EFFECT OF H-ION CONCENTRATION ON THE GROWTH OF *AGARICUS CAMPESTRIS* *

Donald Frear, J. F. Styer and D. E. Haley

(with one figure)

Introduction

It has long been known that *Agaricus campestris*, the common mushroom, grows well on horse manure which has been partially decomposed. In order for fruition to occur, however, the manure must be covered with a thin layer of soil shortly after a good mycelial development has been obtained in the manure. Under these conditions mushrooms develop and mature over a period of several months. At the end of that time, however, the production ceases and before the medium has been depleted of nutrients. The cause of this may be the exhaustion of some food or food material, a change in the physical substratum, the formation of a toxin, or a change in the reaction of the medium. Then again it may be due to a combination of these and possibly other factors. In order to make a study of any single factor affecting the growth of mushrooms, it is highly desirable, if not imperative, to grow mushrooms in pure cultures.

Very little investigational work has been done on the growing of mushrooms in artificial media. Several French investigators, notably Boyer (**1**), have grown the mycelium on agar containing plant decoctions. Boyer found that the best growth was obtained where carrots, finely ground, were used as the basis of the culture. Duggar (**2**), and Ferguson (**3**) also successfully grew mushrooms on synthetic cultures of known composition, although they did not attempt to carry their experiments any farther.

The object of this experiment was to determine, if possible, the optimum H-ion concentration for the growth of the mycelium of *Agaricus campestris* and to determine the effect of the organism upon the reaction of the nutrient medium.

Experimental

The medium used for the growth of the mycelium of the mushroom in question was that used by Styer (**4**), who, in cooperation with Dr. True, of the University of Pennsylvania, found that a fair growth could be obtained with the following mixture:

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* Approved by the Director of the Experiment Station as Technical Paper no. 440.

Black filter paper (Whatman's no. 29)	10.0 gm.
Casein	0.6 gm.
20 cc. of the following solution:	
$MgSO_4 \cdot 7\ H_2O$	0.02 M.
KH_2PO_4	0.04 M.
K_2SO_4	0.01 M.
$FeSO_4 \cdot 7\ H_2O$	Trace
$CaCl_2$	Trace

The differences in hydrogen-ion concentration were obtained by adding varying amounts of acid (H_2SO_4) and base (KOH). One set of cultures (P) was made up using 10 grams of the black filter paper plus 20 cc. of distilled water.

The culture medium was placed in 300 cc. Pyrex Erlenmeyer flasks and the flasks plugged with cotton to avoid contamination, after which they were sterilized in a steam autoclave at 15 pounds pressure and 120° C. for 30 minutes. The flasks were then inoculated by introducing a small piece of mycelium from a pure culture growing in manure. The check flasks were treated in the same manner as the others, except that they were not inoculated.

After inoculation the flasks were removed to a light-proof incubator, which was kept at 27° C. by means of an electric thermostat. The humidity was preserved by means of pans of moist sphagnum moss, in which the flasks were placed. Observations were made and the growth recorded each week in terms of the diameter of the colony in centimeters. The flasks were uniformly 8 cm. in diameter at the base.

The media were extracted with 50 cc. of distilled water, and the H-ion concentration of the resulting solutions were obtained by means of the potentiometer, using Bailey electrodes.

TABLE I

COMPARISON OF THE INITIAL pH OF THE MEDIA WITH THE FINAL pH

CULTURE	pH	
	INITIAL	AFTER 4 WEEKS
C	3.4	3.5
D	3.8	4.6
E	4.1	5.3
F	4.5	4.7
G	5.1	5.0
H	5.5	5.4
I	6.0	4.0
J	6.3	4.3
K	6.7	4.7
L	7.0	5.6
M	8.0	7.6
P	7.0	5.0

The data given in tables I and II were the averages obtained from three cultures grown under exactly the same conditions. The curve of growth with change in pH is shown in fig. 1.

Summary and conclusions

From the foregoing figures it may be seen that the best growth was obtained in the cultures which were nearest to a pH of 6.0, although the organism was tolerant to all conditions which were imposed upon it. The cultures in which the growth was greatest changed the reaction of the media

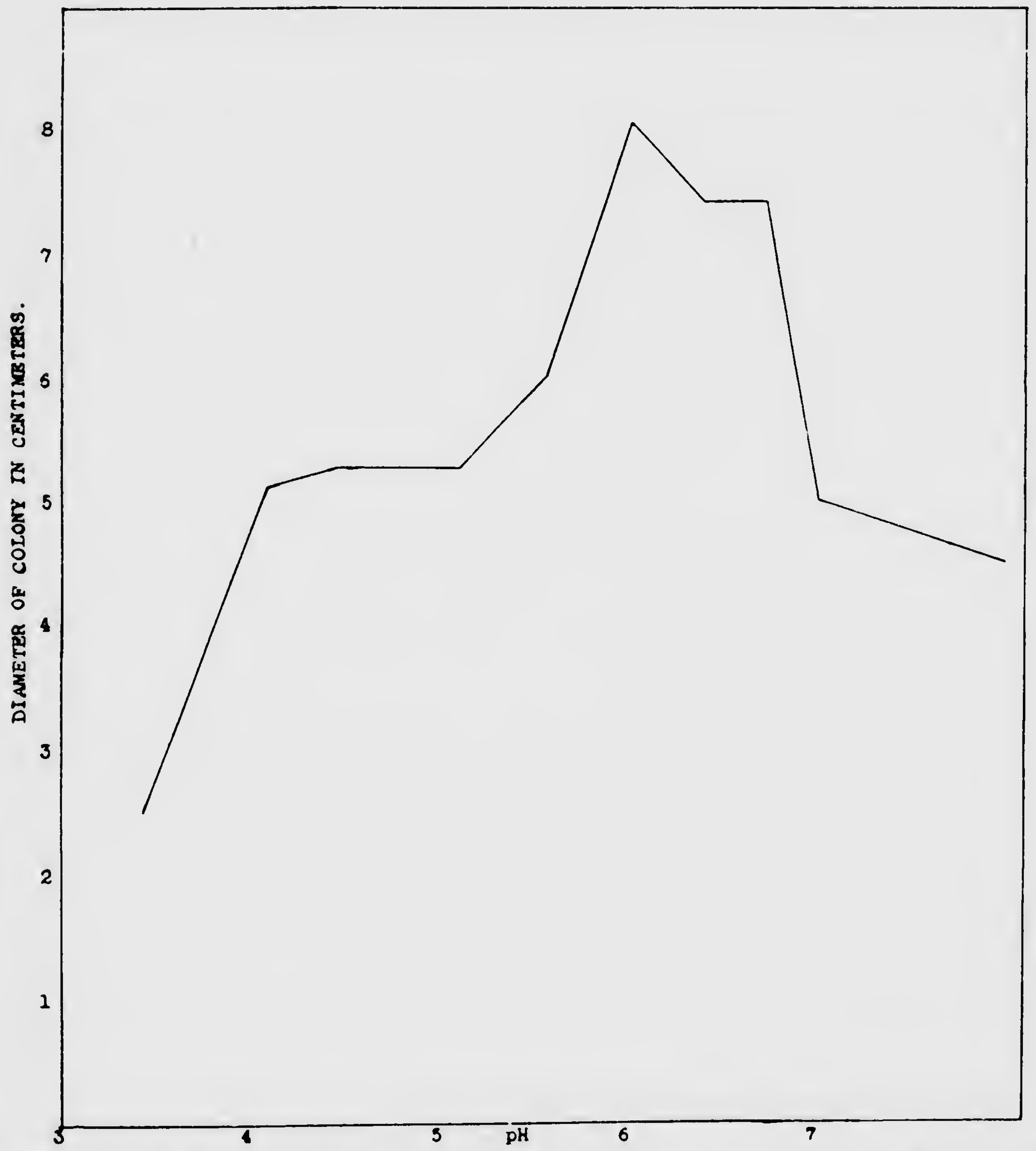

FIG. 1. Growth of *A. campestris* in relation to the H-ion concentration of the medium.

TABLE II

GROWTH OF *Agaricus campestris* AS AFFECTED BY THE H-ION CONCENTRATION OF THE NUTRIENT MEDIUM

CULTURE	pH	GROWTH (DIAMETER IN CM.)			
		1 week	2 weeks	3 weeks	4 weeks
C	3.4	0.4	0.8	2.0	2.5
D	3.8	1.0	3.0	4.2	4.2
E	4.1	1.0	3.0	4.2	5.1
F	4.5	1.0	3.8	4.7	5.2
G	5.1	1.2	4.4	5.0	5.2
H	5.5	1.4	4.6	5.0	6.0
I	6.0	2.0	5.0	7.0	8.0
J	6.3	1.9	5.3	7.0	7.4
K	6.7	1.4	5.6	7.0	7.4
L	7.0	0.5	3.6	5.0	5.0
M	8.0	0.4	2.0	4.0	4.5
P	7.0	0.5	1.0	2.0	2.0

to a marked extent, as evidenced by the marked difference in the initial and final pH in cultures I, J, K, and L.

The authors wish to thank Dr. R. H. TRUE, of the University of Pennsylvania, who suggested this work.

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THE RELATION BETWEEN ELECTRICAL CHARGE AND THE AGGLUTINATING ABILITY OF SALMONELLA PULLORUM[1,2]

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INTRODUCTION

Attention has been repeatedly called to variations in the agglutinability of strains within a bacterial species. This has been especially true of *Eberthella typhi*. Some strains of this species have been encountered which were agglutinated so poorly that they could not be used in the Widal test. On the other hand, the extensive study of Ishii (1922b) shows many hyper- or spontaneously agglutinated strains. Zinsser (1923) considers it a common observation that no two strains of a species are exactly similar in their agglutinability. The various reviews of bacterial agglutination published, especially those of Arkwright (1914); Tulloch (1914); Hooker (1916); Buchanan (1919); Ishii (1922 a and b); Morishima (1921) and Robinson (1923) make another summary of the literature superfluous. Therefore, reference will be made only to the literature which deals with *Salmonella pullorum*.

Extreme variations in the agglutinability of strains of *Salmonella pullorum*, as observed by the senior author, led to the investigation reported in this paper. Recently several investigators have reported similar observations. Mallman (1925) found that

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[1] This is a résumé of a thesis submitted by the senior author in partial fulfillment of the requirements for the degree of Master of Science in Agricultural and Biological Chemistry in the Graduate School of the Pennsylvania State College, 1926.

[2] Published by Permission of the Director of the Pennsylvania Experiment Station as Technical Paper 439.

strains vary in their ability to be agglutinated and questioned the accuracy of the agglutination test unless strains of consistently high titre are used. May and Goodner (1926), after finding considerable variations in agglutinability of strains, concluded that "it seems advisable to select highly agglutinating strains for the routine test for ovarian infection in adult fowls." Gwatkin (1925) has also found that all strains are not agglutinated equally well. It is, therefore, apparent that strains of the species *Salmonella pullorum* may show as much variation in agglutinability as those of *Eberthella typhi.*

It is well to call attention to the fact that the environmental conditions under which bacteria are grown and antigens are produced play an important part in their agglutinability. A strain may vary considerably in its agglutinability when grown on media of different hydrogen ion concentration or sugar content (Ishii (1922a)). The kind and concentration of salts in the suspending menstruum play an extremely important rôle (Northrop and DeKruif (1922)). Therefore, before one can conclude that variations exist between strains of a species it must be shown that all environmental conditions are uniform. It seems that the variations reported, both for *Eberthella typhi* and *Salmonella pullorum* occurred under uniform environmental conditions. It is true that each group of investigators used a different combination of environmental conditions but consideration must be given to the fact that each supposedly maintained uniformity throughout his investigation.

Bordet's (1899) observation that bacteria are not agglutinated by immune serum unless salts are present placed the phenomenon of bacterial agglutination upon a colloidal basis. He showed that specific bacterial agglutination consists of two phases, first, the specific adsorption of agglutinin by the bacteria, and, second, the agglutination or precipitation of the new complex by the salt. Agglutinin, therefore, itself, does not cause agglutination, but when it is specifically adsorbed by the bacterial cells it forms an unstable complex which is precipitated by the action of the electrolyte of the suspending menstruum. This change of behavior resembles the change from a stable hydrophilic colloid to a non-stable hydrophobic colloid which is sensitive to electrolytes.

Many investigators have found that colloids, when suspended in water, are electrically charged. It has also been shown that when placed in an electric field they migrate generally toward the anode, that is, that they are negatively charged. The stability of colloidal suspensions is dependent upon two forces, namely, repulsion and attraction. The mutual repulsion of the cells is due to like charges. On the other hand, surface tension which strives to reduce the free surface by bringing the particles together acts as the attractive force. Both forces are constantly at work, except at an absolute isoelectric point. So long as the repelling force is the greater, the particles remain in suspension but if the electric charge is reduced to a point or zone where the attractive force becomes the greater, precipitation or agglutination occurs.

It has been shown in the summaries of Winslow, Falk and Caulfield (1923) and Winslow and Upton (1926) that the relative charge carried by bacteria can be measured by the relative electrophoretic migration and that suspensions of bacteria as ordinarily grown are negatively charged. Bacteria remain in suspension because of the repelling action of the like electric charges which they carry, an action which opposes the attractive force, or, as Northrop and DeKruif (1922) prefer to say, the cohesive force. These investigators measured both the electrophoretic potential and the cohesive force and found that whenever the potential difference between the surface of the bacterium and the suspending menstruum was reduced to about 15 millivolts the bacteria agglutinated, provided that the cohesive force was not affected. If the cohesive force was reduced, the critical potential had to be lowered and if the cohesive force was made very small no agglutination occurred even if the potential was reduced to zero. They found that bacteria sensitized with immune serum had a constant cohesive force and, therefore, it seemed to us that any difference in the relative ability of strains to be agglutinated must be due to differences in the relative electrophoretic potential, practically to differences in relative electrophoretic velocity.

During the progress of our work Falk, Jacobson and Gussin (1925 a, b and c; 1926 a and b) reported upon a study of the relation between electrophoretic migration velocity, virulence, lethality and agglutinability of the groups of pneumococci. Their results suggest that electrophoretic potential is related in some fundamental manner to agglutinability since they found a sequence of decreasing agglutinability accompanied by a sequence of increasing electrophoretic potential.

OBJECTIVES AND GENERAL RESULTS OF THE PRESENT INVESTIGATION

The investigation reported here was begun during the summer of 1925 to determine, first, if strains of *Salmonella pullorum* vary with respect to their electrophoretic migration velocity, and, second, in case variations were found, to study the quantitative relationship to agglutinability.

Our results show that the species *Salmonella pullorum* bears a negative charge and it is clearly evident that great variations exist between strains so far as quantity of charge is concerned as evidenced by the differences in the rate of electrophoretic migration velocity. Variations were also found in the agglutinating ability of strains. In general, the sequence of decreasing electrophoretic migration velocity was identical with the sequence of increasing agglutinating ability, therefore, there appears to be a quantitative relation between the electrophoretic velocity and the agglutinating ability of strains of this species. This, we believe, explains at least in part, why some strains of a bacterial species are more easily agglutinated than others.

TECHNIQUE

Preparation of the suspension. Proteose-peptone agar adjusted to approximately pH 7.0 has been successfully and conveniently used for the cultivation of *Salmonella pullorum* by the senior author during the past several years and, therefore, was considered suitable for this investigation. In preparing this medium Difco proteose-peptone is substituted for ordinary peptone in nutrient agar. Since Shibley (1924) found that the charge carried by *Esch. coli* varied until the culture was about eighteen hours old, it was deemed advisable to use a constant incubation period of twenty-four hours. The bacteria were grown upon slants at 37°C. The growth was removed with a small volume of 0.85 per cent NaCl by a gentle rotary movement of the culture tube. The suspension was then shaken with glass beads to break up clumps of bacteria. This suspension was filtered through cotton to remove flakes of agar, centrifuged at about 2500 revolutions per minute and the cells resuspended in 0.87 per cent NaCl. The washing was repeated three times to remove traces of peptone, etc., because DeKruif (1921) found that such impurities affected both the quantity of charge and the agglutinating ability. The final washed suspension was heated for one hour at 56° to 58°C. and standardized to a density which corresponded to approximately 3 cm. by the Gates (1920) method. The suspension thus prepared was used for electrophoretic measurements and agglutination tests, both being made the same day that the suspension was prepared.

Measurements of the electrophoretic migration. The apparatus used in this investigation was similar to that devised by Northrop (1922) and of the type now being prepared by the Eimer and Amend Company of New York.

We found that the use of a trace of dye (methylene blue) in the zinc sulphate solution greatly facilitated the filling of the electrode chambers and prevented an excess of zinc sulphate being run into the electrophoresis cell as the colored solution could be seen within the stop-cocks with much greater ease than the colorless one. Before each sample was introduced into the apparatus, the cell was washed with at least one liter of distilled water in order to remove even the slightest amount of zinc sulphate which might have entered when the electrode chambers were filled. During preliminary experiments we found that unless the distilled water was removed completely with NaCl solution of the same concentration as that in which the bacteria were suspended, diffusion currents were produced which prevented the making of readings. Therefore, after the cell was washed with distilled water it was flushed with about 200 cc. of 0.85 per cent NaCl and finally with about 50 cc. of the bacterial suspension.

A direct current of approximately 60 volts was applied, which gave a potential gradient of approximately 2.1 volts per centimeter. Observations were made with direct illumination and a magnification of 525 diameters. All measurements of electrophoretic velocity were made by timing with a stop-watch the migration of a given bacterium across a distance of 32 micra.

Preliminary readings, made in order to determine the depth of the cell and the level of maximum velocity taken at each one-sixteenth of the depth of the cell, when plotted, reproduced the Smoluchowski (1921) curve. This suggested that four readings be made between six-sixteenths and nine-sixteenths of the depth of the cell, each differing by one-sixteenth, because at these levels the migration was fastest. The advantage to be gained by making these four readings, rather than readings at a single level or at certain specific levels as others have done, lies in the fact that a greater confidence is had in the accuracy of the values obtained, for they can be plotted and it can be observed whether or not the curve obtained passes through a maximum. All velocities reported in this paper actually did this very thing. Readings were made upon 20 cells at each level and an average of the 80 readings are reported. The current was reversed after the fifth, tenth and fifteenth readings at each level. We believe that this system of reversing the current is of distinct advantage because even the slightest amount of drifting can be detected easily by unequal migrations in the two directions. The results of our investigation are expressed in terms of micra per second per volt per centimeter since the Helmholtz-Lamb equation used to convert electrophoretic migration velocity into potential difference is questionable as discussed by Winslow, Falk and Caulfield (1923).

Determination of the agglutinating ability. Antisera from two rabbits immunized with strain X-43 were used. The usual procedure for macroscopic agglutination tests was employed. The NaCl needed for making serum dilutions was taken from the same lot as that used to prepare the bacterial suspensions. The serum was diluted to each dilution in large tubes and then pipetted out to the agglutination tubes so that all suspensions would be mixed with serum of exactly the same strength. Readings were made after forty-eight hours incubation at 37°C. The results are presented according to the method advocated by Hadley (1917).

Cultures. The 18 strains used were from widely separated sources and dates of isolation. Half of the strains were isolated by the senior author. A-40 and X-61 were from infected ovaries. X-48 was from the heart of a hen. X-57 was from the heart of a three-month-old chick. All other strains were from either the liver or heart of chicks. All strains were transferred daily for three days before they were used in this work.

EXPERIMENTAL RESULTS

Our first experiments, made to determine the experimental error, showed that almost identical average migration velocities could be obtained when several suspensions were prepared from a strain, as indicated in table 1. These suspensions were prepared as described above but from separate lots of agar slants. An inspection of the data shows that the average migration velocity of any suspension did not vary more than approximately 3 per cent from the average of all suspensions of that strain, thus confirming the accuracy of the method as reported by Falk and Reed (1926).

The results of both electrophoretic measurements and agglutination tests made upon 18 strains are summarized in table 2.

The strains were grouped according to their agglutinabilities. In general, the greatest dilution of serum at which complete agglutination still occurred (titre) was: for group one, about 100; for group two, 800; for group three, 1600; for group four, 3200 and for the homologous strain X-43, 3200. The average electrophoretic migration velocities were: for group one, −1.578; for group two, −1.069; for group three, −0.955; for group four, −0.722 and for the homologous strain, −1.157. This indicates

TABLE 1

Average migration velocities (in micra per volt per centimeter) of repeated experiments

STRAIN	SAMPLE	VELOCITY	AVERAGE OF ALL STRAINS	VARIATIONS FROM THE AVERAGE
X-40	a	−1.123		0.028
X-40	b	−1.068	−1.095	0.027
X-43	a	−1.058		0.008
X-43	b	−1.078		0.028
X-43	c	−1.015	−1.050	0.035

TABLE 2

Migration velocities and agglutination tests

STRAIN	MIGRATION VELOCITY*	AVERAGE OF GROUPS	AGGLUTINATION									SOURCE OF CULTURE
			Serum dilutions									
			1	2	3	4	5	6	7	8	9†	
X-48	−1.714		3	1	0	0	0	0	0	0	0	Heart of hen
B-93	−1.280		4	3	1	0	0	0	0	0	0	Chick
X-57	−1.740	−1.578	4	4	4	2	1	0	0	0	0	Heart of three-month-old chick
A-40	−1.176		4	4	4	4	3	2	1	0	0	Ovary
A-33	−1.138		4	4	4	4	3	2	0	0	0	Chick
X-61	−1.100		4	4	4	4	3	1	0	0	0	Ovary
B-64	−1.019		4	4	4	4	2	1	0	0	0	Heart of hen
A-24	−0.985		c	c	4	4	3	2	1	0	0	Chick
B-104	−0.995	−1.069	4	4	4	4	3	2	1	0	0	Chick
A-12	−0.995		c	c	c	4	4	2	2	0	0	Chick
X-80	−0.964		4	4	4	4	4	2	1	0	0	Chick
B-71	−1.047		4	4	4	4	4	3	1	0	0	Chick
X-49	−0.955	−0.955	4	4	4	4	4	3	1	0	0	Chick
X-52	−0.993		4	4	c	c	4	4	3	0	0	Chick
B-73	−0.646		c	c	4	4	4	4	3	1	0	Chick
B-74	−0.627	−0.722	c	c	c	c	4	4	3	2	0	Chick
X-43‡	−1.157	−1.157	c	c	c	c	4	4	2	1	0	Chick

* Each average migration velocity represents the average of 80 stop-watch readings.

† Serum dilutions 1:100, 1:200, 1:400, 1:800, etc., represented by 1, 2, 3, 4, etc.

‡ Homologous strain.

very clearly a relationship between the electrophoretic migration velocity and the agglutinability of the species *Sal. pullorum*, since there is a sequence of increasing agglutinability which is accompanied by a sequence of decreasing migration velocity. The apparently abnormal behavior of the homologous strain X-43 will be discussed later.

It is interesting to note that strains isolated from mature birds showed relatively poor agglutinability and a high charge.

DISCUSSION

Since electrophoretic migration velocities can be measured to an accuracy of 3 per cent and since a total variation of approximately 63 per cent was obtained, it appears that the variations in electrophoretic results reported are really significant in view of the fact that these variations between strains were observed under uniform environmental conditions, the medium, the 0.85 per cent NaCl, the procedure of making both suspensions and electrophoretic measurements, the pH and the temperature being practically identical in every case.

That the experimental results should reveal relationship between agglutinability and electrophoretic migration velocity was expected as a result of an application of fundamental colloid chemistry. Since bacteria behave as hydrophilic colloids which are changed into hydrophobic colloids by immune serum (Wells (1925)) and since the agglutination of the hydrophobic particles is a question of bringing them into an iso-electric zone (Northrop and DeKruif (1922)), it follows that the charge which is represented by electrophoretic migration velocities must vary in some way with agglutination tendencies. It is, therefore, not surprising, when all experimental conditions except the nature of the organism are kept constant, to find that the series representing the agglutinability and that representing the electrophoretic migration velocities appear to be comparable.

Strain X-43 did not lend itself to a proper placement in the series. In view of the work of Shibley (1924) and the statement made by Falk, Jacobson and Gussin (1926a) namely that "the type specificity of each serum is as plainly evident in the reduction of electrophoretic potential as in the agglutination of the bacteria," it appears that we should not have expected a placement which would have put agglutinability and electrophoretic migration velocity in the same relative position. The placement that was obtained is just that which is to be expected from the above work, namely, better agglutinability than the charge suggests.

That the readings obtained with strains isolated from mature birds showed relatively poor agglutinability and high charge is interesting in view of the work published by Falk, Jacobson and Gussin (1925a) in which they called attention to the fact that, for the pneumococci, charge and virulence paralleled each other. Such a parallelism is suggested here on the assumption that strains from the adults possess more virulence than those from chicks.

Attention is called to the fact that work is now in progress in which an attempt is being made to vary the charge of the organism by varying the components of the medium upon which it is grown, in order to ascertain whether variations in agglutinability and charge can be given to the organism by the constituents of the growth medium to such a degree that they can be carried over when the organism is suspended in various suspending menstra. This work should throw some light on why different strains should have different agglutinabilities and different charge, and on whether work in this field can be duplicated provided workers do not use exactly the same growth and suspending media.

SUMMARY

1. The use of a dye in the solution used to fill the electrode chambers facilitated the filling of the electrophoresis apparatus.
2. The Smoluchowski curve was obtained.
3. It is suggested that greater confidence in readings is obtained by making measurements which will span the hump of the above curve.
4. Electrophoretic migration velocities were determined with an accuracy of 3 per cent.

5. Eighteen strains of *Sal. pullorum* studied carried a negative charge.
6. Variations of over 60 per cent in the electrophoretic migration velocities and a titre ranging from about 100 to 3200 were obtained.
7. In general, the sequence of decreasing electrophoretic migration velocity followed the sequence of increasing agglutinability.
8. The use of homologous serum produced an agglutinability superior to that suggested by the charge.
9. A relationship between electrophoresis, agglutinability and virulence is suggested.
10. Attention is called to work in progress.

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A STUDY OF CERTAIN CONSTITUENTS OF THE LEAF AND THEIR RELATION TO THE BURNING QUALITIES OF TOBACCO[1, 2]

D. E. Haley, E. S. Nasset, and Otto Olson

Introduction

The term "burning qualities" as applied to smoking tobacco, usually includes several different elements. Of these, according to Garner (**9**, p. 8), the fire holding capacity, the evenness and completeness of combustion, and the character of the ash are of most importance. A knowledge of the factors influencing the burning qualities of cigar-leaf tobacco is of considerable importance, both from the standpoint of the producer and the manufacturer, as the value of tobacco for cigar manufacture is based almost wholly upon these qualities. For this reason, therefore, considerable investigational work has been conducted upon this subject, and, as a result, it is now known that several factors, especially those of a chemical nature, influence the "burn" to a considerable extent.

Review of previous investigations

Schlösing (**23**) was one of the first investigators to study the chemical factors affecting the burning qualities of tobacco. He treated tobacco leaves with various salt solutions and found that potassium salts of various organic acids were decidedly beneficial. He attributed their effects to the tendency of these salts to swell on heating, thus exposing more surface of combustible material.

By impregnating filter-paper with solutions of various salts, Nessler (**20**, p. 73) found that the sulphate and carbonate of potassium increased the fire-holding capacity to a marked degree. He also observed that potassium acetate promoted combustibility in a manner similar to the carbonate, although this salt does not possess the power to swell when heated, at least to a marked degree, therefore, Schlösing's interpretation of the catalytic effect of potassium salts of organic acids was probably not correct.

In analyzing the ash of good-burning tobacco, van Bemmelen (**24**) always found a greater quantity of potassium present as the carbonate and lesser quantities as the chloride and sulphate.

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[2] This investigation was conducted in cooperation with Dr. W. W. Garner, of the U. S. Bureau of Plant Industry, Office of Plant Nutrition and Tobacco Investigations, and Professor F. D. Gardner, Department of Agronomy of the Pennsylvania State College.

MAYER (**16**) studied the influence of organic and inorganic substances on the capacity of filter-paper to glow or burn with a flame. The results obtained with inorganic compounds were similar to those obtained by NESSLER. In addition, he found that nearly all organic substances promote burning with a flame.

It has been suggested by BARTH (**2**), that the burn and characteristic glow of a good tobacco is due to a reduction of the alkali salts to oxides and small quantities of free metal, and that such a reduction would increase the efficiency of these metals as oxidizing catalysts.

BEHRENS (**4**), as a result of his investigational work on the burning qualities of tobacco, states that a high content of potassium in combination with citric, malic and oxalic acids largely determines the burning quality. GARNER (**9**, p. 19), is also of the opinion that potassium in such combinations is highly desirable, and further suggests that the favorable action of the carbonate obtained from the combustion of these substances may be due to its effect of functioning as a catalyst in combustion by taking up carbon dioxide and forming the acid carbonate at the most favorable moment and losing it later on. KRAYBILL (**12**), however, obtained results which seem to disprove this theory.

RIDGWAY (**22**) found a striking relationship existing between the fire-holding capacity and the degree of aggregation of various salts normally present in tobacco leaves. Under certain conditions of curing, it appears that there is a noticeable tendency for calcium and magnesium to crystallize as malates, citrates and oxalates. As these bases do not appear to promote the fire-holding capacity to any considerable degree, such a process is desirable, as relatively larger quantities of the tissues are thus left free to burn. He found that where such a crystallization did not take place, the burning quality was always poor.

According to LOEW (**13**, p. 38), the more oxidizable material, and the more oxidation going on within the tobacco leaf, the more will the crystals or "grain" develop in the curing and sweating processes and hence it will in many cases, although by no means in all, confirm the idea of some tobacco manufacturers that a well-developed grain is a good sign of the quality of the tobacco.

That chlorine exerts a deleterious effect on the burning quality of cigar-leaf tobacco is quite definitely established. MAYER (**14, 15, 16**), BEHRENS (**4, 5**), NESSLER (**17, 18, 19, 20**), FESCA (**6**), GARNER (**9**, p. 18), AMES and BOLTZ (**1**, p. 191), OLSON (**21**, p. 11), JENKINS (**11**, p. 95), and others have reported on the poor quality of cigar-leaf tobacco fertilized by chlorine-containing material. JENKINS (**10**) also states that while some chlorine is absolutely essential for the development of the tobacco plant, a large excess may prove deleterious to the burn.

Some work has been done to show a relation between the water-, ether-, and alcohol-soluble constituents of the leaf to its burning quality. GARNER (**9**, p. 18), made water extractions and found that the extracted leaf lost its glowing capacity. On examining the extract, he found that it contained the chloride, sulphate, nitrate, malate and citrate of potassium, ammonium and nicotine, and small quantities of lime and magnesium. Extracts from both good-burning and poor-burning leaves contained about the same quantities of potassium but the inferior leaf contained a much higher percentage of mineral acids. Hence he concluded that the potassium salts of organic acids such as malic and citric are the chief factors controlling the burn. Extraction with alcohol had little or no effect on the burn. It has also been shown by GARNER (**9**, p. 8) that some of the weaker organic acids, on the order of tannic, in combination with nicotine, are deleterious to the burning quality.

GRAHAM and CARR (**7**) made petroleum ether, sulphuric ether, alcohol and hot water extractions of tobacco and showed a relation between poor-burning quality and a high extractive content. They showed that tobacco fertilized with sodium nitrate or muriate of potash yielded a high amount of extractives.

Purpose and plan of the experiment

The object of this investigation was to make a study of the chemical composition of a given strain of cigar-leaf tobacco[3] in relation to its burning qualities, particularly as regards its fire-holding capacity. Particular attention was to be given to the determination of the quantity of ether-soluble organic acids present in cured and fermented samples, and also to their form of combination, as measured by the water-soluble and insoluble alkalinity of the ash. In addition, the effects of chemical treatments and climatic conditions were to be given due consideration.

MATERIALS USED

Eighteen plants, carefully selected from each of ten plots, 1/30th of an acre in area, located at Ephrata, Lancaster County, Pennsylvania, were harvested at the end of the growing season in 1925 and removed to the curing shed, where they were air cured. These samples were taken from the shed at the end of the curing season and dried under laboratory conditions. When they were thoroughly dry, the leaves, stems and stalks were separated, finely ground and transferred to air-tight containers and kept for analysis. This same procedure was followed in 1926.

The chemical treatment of the plots from which the samples were taken is shown in table I.

[3] HIBSHMAN strain, Pennsylvania Broadleaf.

TABLE I

Fertilizer treatments of the experimental plots

Plot	Treatments[a]									
	Manure	Sulphate of potash	Muriate of potash	Nitrate of potash	Manure salt	Bone flour	Precipitated bone phosphate	Acid phosphate	Urea	Ammonium nitrate	Cotton-seed meal
	tons	lbs.	lbs.	lbs.	lbs.	lbs.	lbs.	lbs.	lbs.	lbs.	lbs.
A-1	10	......	200	......	......	250	......	......	......	......	400
A-2	10	200	......	......	......	......	......	300	......	......	500
A-5	10	......	......	......	......	......	......	......	......	......	......
A-6	10	200	......	......	......	......	......	300	......	......	500
A-9	10	......	......	......	400	250	......	......	......	......	400
A-10	10	......	200	......	......	......	......	300	......	......	500
B-1	10	......	200	......	......	......	......	300	65	......	......
B-2	10	......	......	214	......	......	120	......	......	......	......
B-3	10	200	......	......	......	......	......	300	65	......	......
B-6	10	200	......	......	......	......	......	300	......	85	......

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[a] The potash, phosphoric acid and nitrogen-carrying materials, with the exception of manure and cottonseed meal constituted acre applications of approximately 100 lbs. of potash, 48 lbs. of phosphoric acid and 38 lbs. of nitrogen.

Methods of analysis

In determining the total organic acid content of the different samples of tobacco, 10 grams of the material were transferred to a porcelain evaporating dish and 15 cc. of a 20 per cent. H_2SO_4 solution added. The mixture was then stirred thoroughly by means of a spatula, and finally enough powdered pumice stone was added to make the mass appear fairly dry. The mixture was then placed in a paper thimble and extracted with anhydrous ethyl ether for 24 hours. To the ether extract was added 200 cc. of water, the ether allowed to evaporate, the solution cooled, filtered and made up to a volume of 250 cc. Aliquots of 25 cc. were then taken and titrated with 0.04 N. NaOH, using phenolphthalein as an indicator.

In determining the crude ash and alkalinity of the ash, 2 grams of the sample were transferred to a platinum dish and carefully charred, the residue treated with water and filtered. The insoluble portion was placed again in the dish and ignited to constant weight. The filtrate was next added to the dish, evaporated, and the total residue carefully heated, cooled and weighed. The weight of the residue was calculated as crude ash.

About 150 cc. of hot water were next added to the crude ash and filtered. The residue on the filter-paper was washed well and the filtrate obtained was titrated with N/10 H_2SO_4, using methyl orange or xylene cyanal methyl orange as indicator.

The residue on the filter-paper was then placed in a platinum dish, treated with an excess of N/10 H_2SO_4, and the solution heated to boiling and filtered. The excess acid was then titrated with NaOH standard solution and the insoluble alkalinity was calculated accordingly. The usual methods of analysis were employed to determine the remaining constituents.

Experimental

Experiment I

Samples of the 1925 crop were taken and the more important constituents of the web determined. The results obtained are given in table II.

Experiment II

The total ether-soluble organic acid content of the stems, stalks and web portions of the cured samples, and also of the leaves of the fermented samples were determined. In addition, the total water-soluble and insoluble alkalinity of the web portions of the cured material were likewise determined.

The number of cc. of N. NaOH required to neutralize the ether-soluble organic acids in 100 grams of the air-dry material, and the number

TABLE II

The percentage composition of the web of cured tobacco leaves of the 1925 crop as influenced by fertilizer treatment[a]

Plot	Crude ash	SiO_2	P_2O_5	CaO	MgO	SO_3	K_2O	Cl	N	Nicotine
	Per cent.	Per cent.	Per cent.	Per cent.	Per cent.	Per cent.	Per cent.	Per cent.	Per cent.	Per cent.
A– 1	24.08	2.10	0.50	7.88	1.25	1.46	1.89	1.12	4.70	4.89
A– 2	23.79	1.88	0.40	7.06	0.96	1.49	1.94	0.12	4.33	5.14
A– 5	23.99	2.52	0.40	7.30	1.24	1.40	1.93	0.19	4.38	4.61
A– 6	22.78	2.23	0.43	7.58	1.32	1.14	1.65	0.17	4.20	5.16
A– 9	24.15	2.14	0.44	7.93	1.21	1.27	1.95	2.29	4.37	4.56
A–10	23.10	2.23	0.44	7.64	1.15	1.35	1.97	0.95	4.63	4.79
B– 1	24.45	3.53	0.45	7.08	1.16	1.29	1.84	1.26	4.73	4.51
B– 2	23.32	2.09	0.48	7.83	1.31	1.61	1.97	0.19	4.60	4.82
B– 3	23.34	2.22	0.42	7.30	1.08	1.45	1.93	0.22	4.67	4.90
B– 6	23.59	3.87	0.43	7.17	1.14	1.28	2.03	0.25	4.87	4.09

[a] All percentages based on moisture-free material.

of cc. of N. H_2SO_4 required to neutralize the alkalinity of the ash of 100 grams of the same material are given in table IV.

Experiment III

Samples of the 1926 crop were taken after curing and determinations made of crude ash, chlorine, lime, magnesia and potash content of the web portions. In addition, the alkalinity and ether-soluble organic acid content were also determined on the same samples. The results are presented in table III.

TABLE III

The chemical composition of the 1926 crop of tobacco

Plot	Crude ash	Cl	K_2O	CaO	MgO	Alkalinity of ash			Ether-soluble organic acid content
						Sol.	Insol.	Total	
	Per cent.	Per cent.	Per cent.	Per cent.	Per cent.	cc.	cc.	cc.	cc.
A– 1	18.03	0.94	2.13	6.39	0.94	36	293	329	305
A– 2	22.85	0.17	2.29	6.43	0.89	48	283	331	281
A– 5	23.79	0.24	2.55	6.44	0.75	34	278	312	290
A– 6	21.08	0.17	2.38	6.29	0.86	51	323	374	290
A– 9	23.34	1.95	2.69	6.56	0.82	17	333	350	274
A–10	22.80	0.95	2.33	6.79	1.06	37	326	363	293
B– 1	22.78	1.07	2.04	6.83	1.09	25	296	321	286
B– 2	22.92	0.21	2.13	6.79	0.95	40	320	360	293
B– 3	22.54	0.19	3.37	6.75	0.88	39	306	345	283
B– 6	20.70	0.21	2.19	6.78	1.40	32	320	352	300

TABLE IV

The total ether-soluble organic acid content and alkalinity of the 1925 crop of tobacco

Sample	Plant part	Cured material				Fermented material
		Alkalinity of ash			Ether-soluble organic acid content	Ether-soluble organic acid content
		Sol.	Insol.	Total		
		cc.	cc.	cc.	cc.	cc.
A– 1	Web	11	343	354	333	332
A– 1	Stem				234	
A– 1	Stalk				100	
A– 2	Web	39	322	361	336	
A– 2	Stem				300	
A– 2	Stalk				107	
A– 5	Web	22	332	354	347	351
A– 5	Stem				327	
A– 5	Stalk				139	
A– 6	Web	13	343	356	351	345
A– 6	Stem				304	345
A– 6	Stalk				128	
A– 9	Web	8	315	323	314	325
A– 9	Stem				167	
A– 9	Stalk				95	
A–10	Web	13	232	336	331	316
A–10	Stem				223	
A–10	Stalk				106	
B– 1	Web	14	317	331	313	305
B– 1	Stem				225	
B– 1	Stalk				103	
B– 2	Web	19	353	372	362	341
B– 2	Stem				311	
B– 2	Stalk				118	
B– 3	Web	35	309	344	345	324
B– 3	Stem				319	
B– 3	Stalk				163	
B– 6	Web	19	321	340	333	317
B– 6	Stem				324	
B– 6	Stalk				137	

Experiment IV

Samples of the web portions of the cured leaves of the 1925 and 1926 crops were taken and their hydrogen-ion concentration determined by the quinhydrone method (**3**). The results obtained are given in table V.

TABLE V

The hyrrogen-ion concentration of the 1925 and 1926 crops of tobacco

Year	Plots									
	A–1	A–2	A–5	A–6	A–9	A–10	B–1	B–2	B–3	B–6
	pH	pH	pH	pH	pH	pH	pH	pH	pH	pH
1925	5.96	5.96	5.78	5.76	5.97	6.01	5.38	5.74	5.34	5.61
1926	5.95	6.19	6.08	6.16	6.10	6.20	6.37	6.78	6.08	6.27

Experiment V

Burning tests were conducted on strips of the fermented leaves of the 1925 and 1926 crops of tobacco. These strip tests of the fermented tobacco are shown in table VI.

TABLE VI

Strip tests[a] of the 1925 and 1926 crops of tobacco after fermentation

Plot	Burn 1925	Burn 1926
A- 1	Poor	Poor
A- 2	Fair	Fair to good
A- 5	Poor	Poor
A- 6	Good	Good to fine
A- 9	Poor	Poor
A–10	Poor	Poor
B- 1	Poor	Poor
B- 2	Good	Good to fine
B- 3	Fair to good	Good to fine
B- 6	Fair	Fair to good

[a] Definitions of terms in strip tests:
Very poor = tobacco does not hold fire. Coals badly.
Poor = tobacco holds fire but a few seconds. Coals badly.
Fair = tobacco holds fire over five seconds. Coals moderately.
Good = tobacco holds fire evenly over ten seconds. Coals slightly.
Fine = tobacco holds fire evenly until consumed. Coals very slightly.
Excellent = tobacco burns evenly until consumed without coaling and with light colored ash.

Weather conditions

Certain observations were made as to the weather conditions which prevailed during the growing seasons of 1925 and 1926. In 1925 the temperature was relatively high from May 31st until June 10th, and no precipitation occurred until June 25th. July was marked by severe electrical storms and heavy washing rains. The temperature rose gradually during the first two weeks of the month of August. No rain occurred from August 15th to September 13th. The plants were harvested shortly after this period.

The weather conditions for 1926 were quite different. It was moist and cool until June 10th, after which time the temperature rose to a relatively high degree and severe storms occurred during this hot period. The hot weather continued through July and August, but rain storms were quite frequent, breaking all previous rain records for August, and the excessive heat lasted until the plants were harvested. Following are the figures showing the actual precipitation during the growing season of 1926.

Month	Rainfall inches
June	4.31
July	4.57
August	9.12
September	4.47

No accurate measurement of the rainfall during the growing period was made in 1925, but, on the whole, the precipitation was much less than for the season of 1926.

Discussion of results

Experiment I, table II, shows that the chemical composition of the 1925 crop, from an elemental standpoint, was rather constant regardless of the fertilizer treatment. More chlorine, however, was found in the plants receiving chlorine-bearing compounds. The water-soluble and insoluble alkalinity of the ash also varied to a certain extent as shown by experiment II, table IV. The form of potash used appeared to play a part in causing this variation. Where the plants received muriate of potash, the water-soluble alkalinity was less as a rule than where the sulphate was used. A higher total alkalinity was obtained where potassium nitrate was applied. This table also shows a very wide potassium-calcium ratio, a wider ratio than is commonly found in good burning tobacco.

Analyses of the crop of 1926, according to experiment III, table III, show that approximately the same quantity of chlorine was present as in the 1925 crop. On the other hand, the lime and magnesia contents were lower and the potassium content was higher in the 1926 samples, but the potassium-calcium ratio was still too wide for satisfactory combustion. Where chlorine was present in relatively large quantities, a low water-soluble alkalinity of the ash was again apparent. On the whole, however, the water-soluble alkalinity of the 1926 crop was higher than that of the 1925 crop. The principal results obtained from these investigations show, with few exceptions, that there is a correlation between the water-soluble alkalinity of the samples and their burning qualities, and that this effect is more pronounced than that of the relative quantity of ether-soluble organic acids. It appears evident also that the form and amount of potash, and to a certain extent the season, have a great deal to do with the total quantity of water-soluble alkalinity present. If this is true, it is important to account for these effects.

Owing to the limited number of samples studied, it would not be proper to draw many definite conclusions, but it appears that these samples contain too little potassium and too great a quantity of calcium for the most desirable combustion. The data also indicate that the sulphate of potash is more desirable as a carrier of potassium than is the muriate. If potassium functions normally within the plant, it must act as a base. Combined with chlorine or sulphur it does not have the desirable property of neutralizing organic acids to any considerable extent. As chlorine is not normally required in any large quantities by the tobacco plant, it would probably tend to remain in combination with potassium, at least to a certain extent. On the other hand, sulphur may be utilized in relatively large quantities, and under these conditions more potassium would be rendered available for the neutralization of organic acids. As a matter of fact, however, analyses of the plants showed that the sulphur content was not increased by the addition of sulphates, so the reason for the difference in the basic properties of potassium from the sulphate, as compared with the muriate, must be sought elsewhere.

It is a well-known fact that the principal replaceable base of soil colloids, in a calcareous district such as Lancaster County, Pennsylvania, is calcium, and that the addition of potash salts to such a soil should lead to the replacement of calcium from such combinations and the formation of calcium salts. At the same time, it is possible to reverse this process. In other words, there is a competition existing between calcium and potassium, whereby either one or the other goes into colloidal combination. Under laboratory conditions, a sample of soil may be treated with muriate of potash and the replaced calcium may be completely removed by leaching, leaving potassium behind in basic form. On the other hand, it is quite possible to remove all absorbed potassium by the use of a solution of calcium chloride, leaving calcium behind in colloid combination and in basic form. Under natural conditions, neither of the above effects is ever observed, but a relatively dry year, such as in 1925, would have a different effect on the availability of potassium than would a comparatively wet year, as in 1926. Continual leaching of the soil would have the effect of rendering potassium available for absorption in basic form by the tobacco plants, while such a condition would not hold true under relatively dry conditions where soluble calcium salts tend to accumulate in the upper soil layers. Under the former conditions the tendency would be, in the light of our present knowledge, for potassium to assume the carbonate form. Under the latter condition, potassium would tend to assume the form of the muriate or sulphate, as the case may be. These reasons would tend to explain, in a measure, the beneficial effects of a wet season on the burning qualities of Lancaster County cigar-leaf tobacco, but would not necessarily hold true to any appreciable extent on soils lacking in colloidal material, unless the presence of relatively large quantities of calcium tends to inhibit the maximum absorption of potassium by plants.

The beneficial effect of sulphate of potash in a dry year, as compared with the muriate, may be due in part to the difference in chemical activity of the calcium compounds formed where these materials are added, the sulphate being more insoluble and less active chemically than the chloride, and not having the power of replacing potassium, which is possessed by the latter under ordinary conditions.

On the whole, the hydrogen-ion concentration of the 1926 crop was more nearly neutral than the 1925 crop. This would tend to have a desirable effect on the burn in an indirect manner, as it would more nearly approximate the optimum conditions for oxidase activity in the curing and fermentation processes.

Summary and conclusions

This investigation had for its purpose the study of the effect of certain constituents of the leaf in relation to the burning quality of cigar-leaf tobacco. As a result of these studies the following conclusions seem justified:

1. The ether-soluble organic acids of the plant appear to occur almost wholly in combination with the alkali and alkali earth metals, as measured by the water-soluble alkalinity and insoluble alkalinity of the ash respectively.
2. In practically all cases there is a parallelism between the burning qualities and the water-soluble alkalinity of the ash.

3. The season and the form of potash supplied as a fertilizer affected the water-soluble alkalinity of the ash in practically all cases.
4. The phenomenon of base exchange in the soil and the removal of certain active substances through leaching probably have considerable to do with the burn and composition of tobacco, but the difficulty involved in the absorption of potassium, under different conditions, in highly colloidal soils such as those of the experimental plots, should also be given consideration.
5. The lime content of the samples used in this experiment was too high and the potash content too low for maximum burning quality. Further work is in progress relative to increasing the potash content and lowering the calcium content of the tobacco grown in Lancaster County, Pennsylvania.

The authors take pleasure in acknowledging the invaluable assistance of Mr. R. C. Hughes and others who aided in the analytical work.

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THE EFFECT OF POTASSIUM SALTS ON THE AVAILABILITY OF NITROGEN IN AMMONIUM SULFATE[1]

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The data given in table 1 are from certain plots of a fertilizer experiment with vegetables in rotation. This experiment had been in progress for ten years when these summaries were compiled. The rotation consists of early cabbage, early potatoes, tomatoes, and wheat followed by grass and clover in the order named. Complete summaries for the first ten years of the experiment are published in bulletin 210 of the Pennsylvania Agricultural Experiment Station.

TABLE 1

The effect of muriate of potash on yields from ammonium sulfate

CROP	MEAN YIELDS IN TONS PER ACRE						ODDS* BY "STUDENTS" METHOD THAT DIFFERENCES ARE NOT DUE TO CHANCE	
	A	B	C	D	B − A	D − C	B − A	D − C
	Ammonium sulfate and acid phosphate	Sodium nitrate and acid phoshate	Ammonium sulfate, acid phosphate, and muriate of potash	Sodium nitrate, acid phosphate, and muriate of potash	Gain for sodium nitrate with acid phosphate only	Gain for sodium nitrate with acid phosphate and muriate of potash		
Early cabbage	8.73	10.60	7.59	11.42	1.87	3.73	54.5:1	535.0:1
Early potatoes	2.61	2.98	2.54	4.07	0.37	1.53	17.1:1	216.0:1
Tomatoes	10.22	11.13	9.44	14.72	0.91	5.28	4.37:1	132.0:1

* Odds less than 30:1 show that the significance of the difference is questionable.

The respective carriers supplied the following plant-food materials per acre: N, 60 pounds; P_2O_5, 100 pounds; K_2O, 80 pounds.

From these data it appears that the addition of muriate of potash decreased the availability of the nitrogen in ammonium sulfate in these soils. In order to ascertain whether this was true, the following experiment was undertaken. This investigation involves a study of the effects of potassium- and chlorine-bearing compounds, alone and in combination, on the nitrification of ammonium sulfate.

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[1] Approved by the Director of the Experiment Station as Scientific Paper No. 443.

PLAN OF THE EXPERIMENT

Soil was obtained from the strips separating the different tiers of the experimental vegetable plots. These strips were in sod and had received no fertilizer for ten years. One hundred-gram portions of this soil were taken after being screened and mixed with the different chemical compounds according to table 2. The samples were then placed in 150-cc. beakers and the original moisture content of 26.8 per cent was maintained by adding distilled water. Twenty-five series were set up, with 8 replicates in each series.

TABLE 2

Chemical treatments of the different series

BEAKER NUMBER	TREATMENTS—RATE PER ACRE									
	KCl	K_2CO_3	K_2SO_4	KNO_3	$CaCl_2$	$Ca(H_2PO_4)_2$	$NaCl$	$NaNO_3$	$(NH_4)_2SO_4$	NH_4Cl
	pounds	*pounds*	*pounds*	*pounds*	*pounds*	*pounds*	*pounds*	*pounds*	*pounds*	*pounds*
1										
2	160									
3			137							
4		148								
5					236					
6								400		
7									311	
8	160							400		
9	160								311	
10			137					400		
11			137						311	
12						1000		400		
13						1000			311	
14	160					1000		400		
15	160					1000			311	
16			137			1000		400		
17			137			1000			311	
18		148				1000		400		
19		148				1000			311	
20		148						400		
21		148							311	
22				217			275			
23			137							252
24					236			400		
25					236				311	

All applications of K supply approximately the same amount of this element. The same holds true for nitrogen. With the exception of KCl, all chlorine treatments carry the same quantity of chlorine as used in the form of NH_4Cl.

The beakers were covered with opaque cardboard which was weighted with glass panes, and above these was placed moist sphagnum moss. The whole was kept in a dark, well-aerated chamber. Moisture was added as lost from time to time. At the periods stated in table 3, two beakers were taken from each of the different series and the nitrate content determined by the phenoldisulfonic acid method. At the end of 7 weeks the soil in the beakers then remaining was taken out, mixed thoroughly and returned to the beakers, and brought to the proper moisture content. This was done in order to improve the aeration of the samples.

TABLE 3

Nitrate production as modified by chemical treatment

NUMBER OF BEAKER	TREATMENT	QUANTITY OF NO_3 PRODUCED			
		3 weeks	5 weeks	7 weeks	10 weeks
		p.p.m.	*p.p.m.*	*p.p.m.*	*p.p.m.*
1	Nothing	6.4	10.0	40.0	38.9
2	KCl	9.1	14.7	25.1	37.0
3	K_2SO_4	13.3	18.6	25.4	40.2
4	K_2CO_3	6.3	14.1	22.5	41.7
5	$CaCl_2$	−3.3*	7.4	18.0	44.9
6	$NaNO_3$	15.4	15.4	7.1	45.3
8	$NaNO_3 + KCl$	15.4	11.1	14.3	43.2
10	$NaNO_3 + K_2SO_4$	−6.9	11.7	20.7	42.1
20	$NaNO_3 + K_2CO_3$	42.7	0.6	3.3	54.7
24	$NaNO_3 + CaCl_2$	−11.5	−4.8	8.3	50.0
12	$NaNO_3 + CaH_4(PO_4)_2$	10.5	11.7	27.4	42.9
14	$NaNO_3 + CaH_4(PO_4)_2 + KCl$	7.7	9.4	13.2	42.5
16	$NaNO_3 + CaH_4(PO_4)_2 + K_2SO_4$	15.0	1.0	14.5	34.4
18	$NaNO_3 + CaH_4(PO_4)_2 + K_2CO_3$	−6.4	0.0	28.9	52.4
7	$(NH_4)_2SO_4$	28.3	40.6	40.9	64.8
9	$(NH_4)_2SO_4 + KCl$	37.1	33.1	51.3	65.4
11	$(NH_4)_2SO_4 + K_2SO_4$	17.2	34.6	33.6	65.7
21	$(NH_4)_2SO_4 + K_2CO_3$	18.7	20.3	33.3	67.5
25	$(NH_4)_2SO_4 + CaCl_2$	21.4	15.0	34.4	69.3
13	$(NH_4)_2SO_4 + CaH_4(PO_4)_2$	38.6	35.3	40.3	70.9
15	$(NH_4)_2SO_4 + CaH_4(PO_4)_2 + KCl$	37.4	30.7	36.7	75.3
17	$(NH_4)_2SO_4 + CaH_4(PO_4)_2 + K_2SO_4$	35.7	29.4	31.6	68.1
19	$(NH_4)_2SO_4 + CaH_4(PO_4)_2 + K_2CO_3$	33.8	25.1	28.7	89.4
22	$NaCl + KNO_3$	−17.3	−3.7	12.8	40.3
23	$NH_4Cl + K_2SO_4$	6.8	16.1	34.5	60.7

* Minus signs indicate a decrease below the quantity of NO_3 present in the soil at the beginning of the experiment.

RESULTS OF THE EXPERIMENTS

The amounts of nitrates present at the end of stated intervals are given in table 3. The quantity of nitrates is expressed in parts per million of the extract obtained by shaking 100 gm. of the soil with 500 cc. of water, allowing to stand for 20 minutes, and filtering. The results are corrected for the amount of nitrate present in the soil at the beginning of the experiment, and for nitrates added as part of the chemical treatments. The values given are averages of two beakers from each treatment, which in practically all cases were in very close agreement.

SUMMARY AND CONCLUSIONS

As shown in table 3, neither chlorine nor potassium compounds consistently inhibited the process of nitrification. In fact, in soils treated with ammonium sulfate, the rate of nitrification was greater at the end of the test for all additions of either chlorine or potassium compounds. The direct effect of potassium chloride on nitrification is evidently not the explanation of the situation presented in table 1.

Very rapid nitrification during the first three weeks was generally followed by a loss of nitrates during the following two weeks. Mixing the soil at the end of seven weeks resulted, on the whole, in increasing the rate of nitrification. This fact is noteworthy in view of its possible significance to future studies on the effects of fertilizers on nitrification.

Interchange of ions, $NaCl + KNO_3$ and $NH_4Cl + K_2SO_4$, instead of $NaNO_3 + KCl$ and $(NH_4)_2SO_4 + KCl$, respectively, decreased the rate of nitrification. The difference for the NO_3 interchange was practically the same as for the SO_4 interchange.

As shown in table 3, all fertilizers increased the rate of nitrification during the last three weeks, as compared to the unfertilized check. In the same period, potassium carbonate caused a higher rate of nitrification than either potassium chloride or sulfate. Potassium carbonate was associated with the largest quantity of nitrates present for any combination, both at the end of three weeks and at the close of the experiment.

Mono-calcium phosphate, during the last three weeks, tended to decrease the rate of nitrification for combinations containing sodium nitrate, but increased the rate of combinations with ammonium sulfate.

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VITAMIN B TERMINOLOGY

IN November, 1927, Dr. E. V. McCollum, president of the American Society of Biological Chemists, received the following communication from the British Committee on Accessory Food factors:

Dear Sir:

At a meeting of the Accessory Food Factors Committee (appointed jointly by the Lister Institute and the Medical Research Council, 1918) and others interested in vitamin research in this country,[1] which was held on November 14, 1927, at the Lister Institute, under the chairmanship of Sir Frederick Gowland Hopkins, a discussion took place upon the nomenclature of vitamin B and its recently defined constituent parts.

The committee feel strongly that those workers in this field of research, should, if possible, arrive at some provisional general agreement regarding this point, so that references in the literature emanating from different schools may be uniform and unnecessary complications may be avoided.

The following suggestions were unanimously supported by those present: (1) That pending further investigation, much confusion will be prevented if the designation vitamin B, first used by McCollum and Davis and now firmly established in the literature, should be retained for all dietary factors belonging to this group of water-soluble vitamins. (2) That the term vitamin B_1 should be adopted for the antineuritic (more heat-labile) water-soluble dietary factor which was first discovered by Eykman in 1897 and is required to prevent polyneuritis in birds and marasmus with, or without, paralysis in mammals and beriberi in man. (3) That the term vitamin B_2 should be adopted for the more heat-stable, water-soluble dietary factor, recently described and named P–P ("pellagra-preventive") factor by Goldberger, Wheeler and Lillie and Rogers (1926) and found necessary for maintenance of growth and health and prevention of characteristic skin lesion in rats, and considered by the latter workers to be concerned in the prevention of human pellagra.

Such a scheme as the above possesses the advantage that place could conveniently be found to include any other constituents of this group which might be discovered subsequently.

The committee was of the opinion that the term "Bios" should be retained to denote the factor or factors encouraging rapid growth of yeast cells.

The committee have instructed me to forward the above suggestions to the American Society of Biological Chemists in the hope that they may be brought to the notice of its members and may invite their comments and criticism. It is held that all will agree upon the necessity of reaching agreement upon this point.

A copy of this letter has, at the same time, been forwarded to the British Biochemical Society and to some of the principal workers in this field of research on the continent and in the United States of America.

I am, yours faithfully,
(Signed) HARRIETTE CHICK,
Secretary

In response to this communication President McCollum appointed (in December, 1927) a Committee on Vitamin B Nomenclature to report at the April meeting of the American Society of Biological Chemists. This committee consisted of H. C. Sherman (Columbia University), Atherton Seidell (Hygienic Laboratory, Washington, D. C.), P. A. Levene (Rockefeller Institute for Medical Research), Harry Steenbock (University of Wisconsin) and R. Adams Dutcher (Pennsylvania State College).

Opinions of several interested workers, in addition to the members of the committee, were sought and letters were received from others. A total of fifteen workers have written the committee expressing their views. In many cases the opinions expressed represented the opinions of from one to eight coworkers.

The opinions expressed by the various groups of American workers were far from unanimous, the majority voting unfavorably on the suggestions of the British committee, except for the suggestion regarding the retention of the term "Bios." No letters have been received expressing opposition to this suggestion. Analysis of all suggestions submitted indicates that American workers favor three different systems of nomenclature for the components of the vitamin B complex.

In brief the three systems of terminology suggested may be described as follows:

(1) *The British suggestions:* This terminology would use the letter B to designate the complex, B_1 to identify the heat-labile factor, and B_2 to refer to the heat-stable factor or factors which have to do with appetite and growth stimulation, amelioration of skin affections, etc.

(2) *The Sherman suggestions:* Dr. Sherman suggests the term F to designate the heat-labile factor and G to identify the heat-stable fraction.

(3) *The McCollum suggestions:* Dr. McCollum suggests that the term B be restricted to refer to the heat-labile factor, since it was the first one studied,

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[1] Sir Frederick G. Hopkins, F.R.S., *chairman;* Professor J. C. Drummond; Professor A. Harden, F.R.S.; Sir Charles Martin, F.R.S.; Professor E. Mellanby, F.R.S.; Professor R. H. A. Plimmer; Professor R. A. Peters; Dr. Harriette Chick, *secretary.*

and to create new letter designations for subsequent components of the complex as fast as they are recognized as separate entities. He suggests the letter F (or G) for the heat-stable factor. Other suggestions were made, but since they were not supported by many workers, reference to them will be omitted.

It is impossible to do more than summarize the arguments of the proponents of the various systems of nomenclature. The letter from the British committee, quoted above, will give the reader the reasons for their suggestions.

On January 10, 1928, Dr. Sherman wrote the following letter in explanation of his suggestions:

> (1) I concur in the recommendation of the British Committee that the term "Bios" be retained as stated.
>
> (2) I concur in the implied recommendation of the British Committee that no one substance should hereafter be designated by the unqualified term "Vitamin B."
>
> (3) For the two now recognized substances formerly covered by the term vitamin B, I think that the designations "Vitamin F and G," proposed somewhat over a year ago in the *Journal of Chemical Education* and also last summer in the *Journal of Biological Chemistry*, are much preferable to the designation B_1 and B_2 proposed by the British Committee.
>
> To discuss all the advantages which, in my judgment, are possessed by the designations F and G over B_1 and B_2 would make this letter too long. A few of them are as follows:
>
> (a) *Consistency with the accepted plan of designation* of the vitamins by letters, according to which each substance of this group, when its existence has been sufficiently demonstrated, receives as designation a separate capital letter, assigned in alphabetical sequence as the need for each becomes clear. The designations F and G are entirely consistent with this established and accepted plan, while the designations B_1 and B_2 are not consistent with it, since they use the same letter for different substances.
>
> (b) *Definite freedom from unwarranted implications* in the case of the designations F and G, whereas the designations B_1 and B_2 imply (would certainly suggest to many if not most readers) that these two substances are more closely related to each other than are any other two substances of the vitamin group (vitamins A and D, for example), an implication which may or may not prove true but for which there is I think no adequate evidence at the present time.
>
> (c) *Very great advantage in presentation* to students and to the public. The reason for having new designations is that we are satisfied that the two factors are separate substances. This is clearly indicated by the use of the terms F and G, whereas the use of the terms B_1 and B_2 might easily lead to the supposition that they are merely two forms or phases of the same substance. Designations involving subscripts are awkward to speak, to typewrite or to print. The frequency with which we see ultra-violet rays referred to as "violet rays" in the newspapers should warn us that in practice the subscripts would often be slighted and either B_1 or B_2 would be liable to appear as "vitamin B" in a newspaper or other popular presentation (as well as in students' notes). And still greater would be the confusion if we should later have a B_3 and perhaps a B_4, whereas if we adhere to the plan originated by McCollum, developed by Drummond, and uniformly followed hitherto, *viz.*, that of giving *each recognized vitamin a separate capital letter*, there should be little danger of confusion either in technical or non-technical discussions.

In a recent letter, Dr. Sherman expresses the opinion that he is not interested in retaining the term B to denote the complex. In fact he feels that nothing would be lost if the term B should be given "honorable retirement," leaving this space blank in the system of vitamin nomenclature.

Sybil L. Smith has already advanced arguments for Dr. Sherman's suggested system of terminology.[2]

The following letter of Dr. McCollum, written on February 24, advances arguments in support of a third method of terminology:

> (1) Each vitamin principle essential to the rat should be recognized as a separate entity; each should receive a separate name. There should be no attempt at grouping them into an artificial complex, that is, subordinating them to a generic name. The substances should be treated as individual factors of equal rank.
>
> (2) Provision for vitamins which may be discovered in the future but which are at present unknown.
>
> The terminologies proposed by Goldberger, Salmon and Chick and Roscoe are open to criticism because they violate the first criterion. They take the unnecessary, indeed, misleading step of supplying the complex with a name, implying a subordinate rôle for each factor of the complex. The recommendations of the Accessory Factor Committee show even more subordination of the independent substances not only in assigning a generic name to their artificial complex, but also in giving subnumerals to the constituent factors. In addition, the numerals possess no advantage over the generally accepted system of letters. The same criticisms may be levelled against figures as against letters, but usage has made adherence strictly to letters very desirable.
>
> The system that meets the criteria most exactly is Drummond's modification of McCollum's original plan. This deals with each vitamin as an entity; there is no grouping or subordination. Further, it provides for additional members as rapidly as they are recognized.
>
> We have seen that at present only two factors, the thermo-labile antineuritic principle, and the thermostable anti-pellagrous principle need identification. Adhering to the McCollum-Drummond plan involves a new use for the term vitamin B as a definite factor, else it becomes a blank in the scheme. It can not be used in its old sense.
>
> It is proposed that:
>
> (1) The term vitamin B be adopted for the antineuritic (more heat-labile) water-soluble dietary factor which was first discovered by Eykman in 1897 and is required to prevent polyneuritis in birds and marasmas, with or without paralysis, in mammals, and beri-beri in man.
>
> (Vitamin B even in its older sense has been generally regarded as thermo-labile, and the antineuritic effect has long been associated with this term. For these reasons the thermo-labile antineuritic principle rightfully merits the priority to the term vitamin B. In this proposed scheme vitamin B has merely a more restricted meaning than formerly.)
>
> (2) The term vitamin F (or G) should be adopted for the more heat-stable water-soluble dietary factor described and named by Goldberger, Wheeler, and Lillie and Rogers as factor P–P, and found necessary for maintenance of growth and health and prevention of characteristic skin lesions in rats, and considered by the latter workers to be concerned in the prevention of human pellagra.
>
> (This factor was discovered as an independent principle by Goldberger, *et al.*, and should be treated as a separate vitamin, taking the next available letter in the alphabet, namely F (or G).)
>
> (3) No generic name shall be given to all dietary factors belonging to this group of water-soluble vitamins, since it is not only unnecessary but also misleading. In fact, mention of "complex" should be abandoned.
>
> (Vitamins B and F (or G) are independent factors on equal rank with all other vitamins, and need no collective term.)
>
> (4) Recognition of existence of additional water-soluble vitamins in the future shall be met by taking the next available letter in the alphabet, following the usual prefix vitamin.
>
> (5) Bios shall be retained to designate the factor or factors encouraging rapid growth of yeast cells.

Under date of February 6, 1928, Dr. Steenbock advanced the following reasons for his preference:

> Previous to the organization of the committee, I was in receipt of the report of the British Accessory Food Factors Committee to which my reaction was very unfavorable. To include the heat-labile and the heat-stable factors, and possibly others, under vitamin B differentiated by subscript suggested to me a common parentage or at least a greater relationship between them than appears justifiable on the basis of present knowledge. If the precedent of such a system were once established for any one vitamin, then it would soon be used for others. For instance, I see no reason why it would not be quite justifiable to use such a system in connection with vitamin A and the antirachitic factor.
>
> When I read Dr. Sherman's suggestions in the *Journal of Chemical Education* that the term vitamin B should be used for the B *complex* and that heat-labile and heat-stable factors should be designated respectively vitamin F and G, my reaction was again unfavorable. In the first place, I see no reason why a vitamin complex of variable quantitative composition should be designated by a letter and, in the second place, I see no reason why the heat-labile antineuritic factor should not be accepted as vitamin B. If historical back-ground counts for anything, it may be recalled that it was McCollum's belief that his water-soluble vitamin B was identical with Eykman's antineuritic principle. Why not continue the use of the term vitamin B in this sense irrespective of its relationship to growth and then use the letter F (or G) for the designation of the heat-stable P. P. factor of Goldberger.
>
> I quite agree with the opinion expressed by the British Accessory Food Factors Committee that it is advisable that the term Bios be retained. In fact I see no reason why other names of suitable designation should not be used by various investigators in their own field of investigation until accumulated evidence warrants the inclusion of the newly discovered factor among the vitamins needed by the animal. It seems to me that it would be expecting too much to have all substances of the nature of vitamins which are indispensable for different types of life to be immediately included in the alphabetical system of nomenclature. When it is found that such substances are indispensable for animals their final inclusion in this system could then be decided upon by a committee on vitamin nomenclature reporting in this country to the Federation of American Societies for Experimental Biology.
>
> From the above you can see that my ideas are quite reactionary to contemplated changes. In fact I am quite convinced that such confusion as threatens us is due not to inavailability of a proper system of nomenclature, but to a paucity of experimental facts and to a certain hastiness in naming. The former will ultimately correct itself, the latter can be corrected by delegation.

Many workers merely wrote that they favored one system or another, without detailed reasons for their opinions. Dr. Sybil Smith's letter in support of the Sherman suggestions has already been published, in substance, and will not be included here.

Nutrition workers will also be interested in the following quotation from Dr. H. H. Mitchell's letter of April 10, 1928.

> There is no question but that the situation with respect to the nomenclature of the components of vitamin B is confusing. The use of the letter B has been so generally applied to what was thought to be a definite dietary factor with growth-promoting and antineuritic properties, that its entire elimination from the list of vitamin letters seems advisable to me. Even its use to denote the combination of the two or more water-soluble components, as has been suggested, seems objectionable to me, since it would refer to no definite combination of

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[2] SCIENCE, lxvii, No. 1741, 494, May 11, 1928.

dietary factors and hence would have no definite significance. I can not conceive of any situation requiring the use of such a loose term.

The A and D situation was not analogous to the B situation, since, in this country at least, vitamin A was not at all generally confused with the antirachitic factor, so that the discovery of this factor, although possibly vitiating some of the work done on vitamin A, did not modify the meaning generally attached to this term.

I favor the use of new letters to designate the components of vitamin B, but I feel that there is danger of making the same sort of an error in defining vitamins F and G as was made in defining B, with the same unfortunate confusion of ideas and the same misdirected experimentation. There is such a general feeling of satisfaction in the establishment of the identity of two factors in any scientific problem, because its ultimate solution is to that extent simplified, that the pronouncement of such an identity is frequently not subjected to the critical scrutiny that its importance deserves. In the present case, there is nothing to be gained, and the possibility of considerable confusion is incurred, by identifying vitamin F (investigated in rat-feeding experiments) with the antineuritic vitamin and, particularly, vitamin G with the antipellagra vitamin, if such there is, until the evidence of these identities is established beyond a reasonable doubt. I think Sherman's statements on this matter are premature.

To my way of thinking it is time, in these days when pronouncements of new dietary factors are becoming so frequent, to come to some general agreement concerning the proper criterion for the demonstration of a new vitamin. Surely the statement that a new vitamin exists should be something more than the mere expression of one's inability to explain experimental findings on any other basis. The factor should be obtained in a potent and concentrated form, the addition of which in minute amounts to a ration, preferably synthetic in character but in all cases demonstrably complete in all other known respects, will *invariably* induce a marked betterment in the nutritive condition of properly prepared experimental animals, an effect which is not dependent upon an increased intake of food. Some such criterion should be satisfied, preferably by more than one investigator working independently, before a letter in the vitamin series is assigned to a new dietary factor. Conservatism in such matters is the wisest policy to pursue.

It would appear that a majority of workers, who have indicated a preference, are quite agreed (in the light of Dr. Herbert Evans' introduction of vitamin F) on the use of the term "G," to denote the heat-stable factor. Some difference exists, however, relative to the term which should be used to designate the heat-labile antineuritic factor.

The committee is not ready to make its final report and it will welcome suggestions from all interested workers in the vitamin field. Steps are now being taken to cooperate with other scientific groups before making definite recommendations.

The members of the committee feel that the naming (by other than descriptive terms) of newly discovered food factors should be discouraged until a system of terminology has been agreed upon. Many workers have expressed themselves as being in favor of discouraging the designation of new vitamins until their identity has been established beyond question. In this the committee is in accord.

Suggestions have been made that an American committee be appointed, representing the interested scientific societies, which may act as a clearing house for questions in vitamin terminology and perhaps cooperate with similar foreign committees in actually naming new factors. This suggestion is made with the hope that it will avoid confusion in the literature and promote uniformity, which is highly desirable.

R. ADAMS DUTCHER, *Chairman*,
Committee on Vitamin B Nomenclature, American Society of Biological Chemists, Pennsylvania State College.

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SYNTHESIS OF VITAMIN B IN THE RUMEN OF THE COW.*

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(Received for publication, September 14, 1928.)

In our studies concerning the vitamin B requirement of calves, one of us produced conclusive evidence that a calf will grow normally to maturity and produce normal offspring on a ration that carries an insufficient amount of the vitamin B complex to support growth and well being in rats (1). In later investigations we determined that vitamin B in milk is not dependent on the presence of this vitamin in the ration of the cow (2). Three cows that were fed for over 2 years, throughout their growth period, on a ration that was decidedly deficient in vitamin B, were used in this study. The evidence obtained in these investigations appeared to indicate that cattle, and possibly all other ruminants, possess the ability to synthesize vitamin B. Damon (3), Kuroya and Hosoya (4), Heller *et al.* (5), Scheunert and Schieblich (6), Sunderlin and Werkman (7), and others have made contributions to our knowledge of the possible synthesis of vitamin B complex by bacteria. The present paper is intended to set forth the results of an investigation which was designed to determine whether the microorganisms present in the rumen of one of our experimental cows were responsible for the synthesis of vitamin B complex.

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EXPERIMENTAL.

A pure bred Holstein heifer, Penstate Homestead Jessie, No. 924062, was made the subject of this study. She was one of a group of seventeen experimental animals, and had grown to maturity on the experimental ration, which was made up from corn gluten meal, cane sugar, commercial casein, polished rice, corn-starch, pearled hominy, dried sugar beet pulp, cod liver oil, and mineral supplements. This ration was demonstrated, through rat feeding trials (1), to be practically devoid of the vitamin B complex.

A permanent fistula about $3\frac{1}{2}$ inches in diameter was made in the rumen of the experimental heifer through the left side. This provision afforded an easy means of sampling the rumen contents. A special pack, after the method used by Schalk and Amidon (8), was provided for keeping the fistula tightly closed so that normal conditions within the rumen would be maintained. The heifer continued to thrive (on the ration deficient in vitamin B) and gave every evidence of functioning normally as she had done on the experimental ration, prior to the operation.

Our first step in the investigation consisted in testing out alcoholic extracts from the fermented rumen contents. 12 hours after feeding, portions of about 30 pounds of the wet fermented feed were removed from the rumen and placed in a 5 gallon milk can. The fermented material was incubated for 5 days at a temperature of 37°, with occasional stirring of the mixture in order that the bacterial flora might multiply and produce, if possible, the maximum quantity of vitamin B. This procedure was considered preliminary, and precautions were taken to provide an extract sufficiently concentrated to eliminate possible difficulties in feeding technique. A total of 56,530 gm. of the fermented rumen contents, representing four separate samplings, was used in preparing the extract. This represented 6252 gm. of dry matter, the average percentage of dry matter being 11.06 per cent.

The fermented rumen contents were stored in 5 gallon stone jars. 95 per cent ethyl alcohol was added to the jars in liberal amounts because of the large amount of moisture present. It was the aim to provide a solution of approximately 70 per cent alcohol. The jars containing the mixture were allowed to stand for 1 week at room temperature with occasional stirring each day.

The liquid was then filtered off and evaporated to dryness at a temperature of 50°, after the usual practice of preparing vitamin B extracts. 2226 gm. of extract carrying 1200 gm. of dextrin, were so prepared that 1 gm. of this extract represented 25.4 gm. of the original fresh fermented rumen material.

Rat Feeding Experiments with Rumen Extract.

The feeding technique used in this and later feeding trials is essentially that described by Dutcher, Francis, and Combs (9). The animals were placed on experiment when they were 21 days of age, and their average weight was about 40 gm. Males and females were used in equal numbers, and litter mates were distributed throughout the groups. Each rat was kept in an individual cage provided with a screen bottom (10). Growth records were obtained and food intake noted.

The animals refused to eat the ration containing rumen extract for the reason that it carried a decided butyric acid odor. 3 per cent of olive oil incorporated in the ration proved to make it palatable. The ration finally adopted (Ration 103) was made up as follows:

	per cent
Casein	18
Salts 185*	3
Agar	2
Olive oil	3
Dextrin	24
“ rumen extract	50
	100

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* McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1917, xxxi, 181.

The controls also receive an equivalent of olive oil in their ration. Each rat was given 4 drops of Squibb's cod liver oil daily to insure an adequate supply of vitamins A and D.

The average growth curve for the two groups is shown in Chart I. In the 8 week period the groups receiving the rumen extract made an average gain in weight of 5.2 gm. per week (Curve I).

The growth curves indicate clearly that the rumen extract must have supplied the vitamin B complex since all animals maintained a constant rate of growth throughout the 8 week period. The controls (Curve II, Chart I) were significantly different although they continued to live (some of them for as long as 10 weeks), but their increases were very slight during the 8 week period.

The evidence obtained in this feeding experiment seemed to indicate quite clearly that vitamin B must have been synthesized by bacteria or other microorganisms. The next step was to make a study of the microflora in the rumen.

Bacterial Study of Rumen Microflora.

The work of Damon (3) on acid-fast bacteria which synthesized the vitamin B complex, suggested that we might be dealing with a similar situation in the rumen of our experimental cow. We had also expected to find wild yeasts. Direct microscopic examination of stained smears of the rumen contents revealed the presence of neither acid-fast bacteria nor yeasts. Samples plated with plain nutrient agar revealed approximately 2,225,000 bacteria per gm. of rumen contents but no yeast colonies were found. What we did find was an almost pure culture of a bacterium that produced a small colony about 1 to 2 mm. in diameter. The colonies developing upon the surface showed a pale lemon-yellow pigment. The subsurface colonies developed no pigment but when transfers were made to agar slants, they proved to be identical with the surface colonies. A search of the literature revealed that our organism had never been described. We have, therefore, designated it *Flavobacterium vitarumen.* Platings on plain nutrient agar of several samples showed the results indicated in Table I. The predominance of this type of colony in our first plates prompted us to make preparations for a test of its ability to synthesize the vitamin B complex.

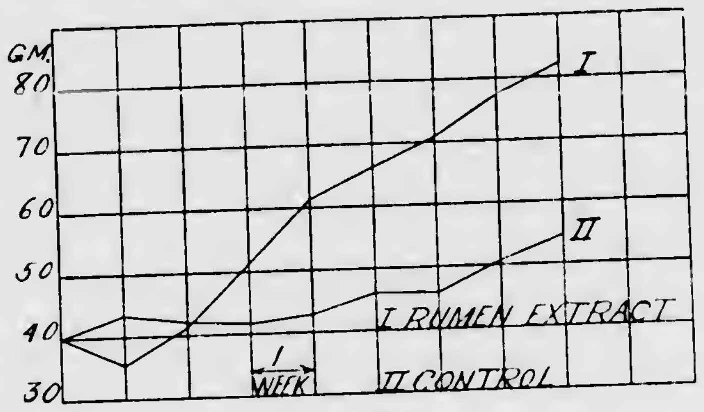

CHART I.

Plain nutrient agar medium, made from the dehydrated Bacto-Nutrient Agar manufactured by the Digestive Ferments Company, was placed in 32 ounce bottles of the Blake type and sterilized for 20 minutes at 15 pounds pressure. The bottles were placed in a horizontal position in order that the agar would solidify on one side of the bottle and provide the maximum area for growth. The media in these bottles were then inoculated with a water suspension of a pure culture of the organism and incubated for 48 to 72 hours at 37°. The growth, which was not abundant, was then washed from the surface of the media with sterile water and the washings were evaporated to dryness at a temperature of approximately 50°. The amount of growth secured in this manner was less than 1 gm. of dried material per bottle. We were able, however, to prepare a sufficient quantity of dried bacterial cells in the form of a meal to make the necessary test for vitamin B in the rat feeding experiments.

TABLE I.

Date.	Total No. of bacteria per gm.	Approximate percentage of *Flavobacterium vitarumen.*
Feb. 21	2,225,000	98
" 26	8,500,000	98
Mar. 4	1,840,000	97
" 17	1,850,000	96
June 30	26,300,000	95
July 13	27,450,000	90
Aug. 1	34,500,000	50

Flavobacterium vitarumen is rod-shaped, being 0.5 to 1.5 microns × 0.5 – 3.0 microns in size. It has rounded ends, does not form endospores, generally occurs singly but occasionally in pairs, and is non-motile. It differs from the vast majority of the genus *Flavobacterium* in that it is Gram-positive.

Surface agar colonies are 1 to 4 mm. in diameter, while the imbedded colonies are somewhat smaller. Gelatin colonies are very similar to the agar colonies. Other cultural characteristics are as follows: Agar slant, filiform; chromogenesis, pale lemon-yellow; gelatin stab, no liquefaction; sugar broths, no gas produced; sucrose, dextrose, and maltose broth, acid produced;

lactose, xylose, dulcitol, mannitol, sorbitol, and insitol broth, no acid produced; starch agar, no diastase; cellulose, no fermentation; lead acetate agar, no growth; Endo's medium, no growth.

Litmus milk is fermented with the production of acid. There is a slight reduction of the litmus but no curd is formed.

It does not form a ring of pellicle growth on any of the broth media. The growth clouds the liquid and finally settles. It does not form indole or skatole and nitrates are reduced.

Rat Feeding Experiments with Dried Bacteria.

The feeding technique was the same as that employed in the previous test on the rumen extract. The dried bacterial meal was fed daily in 0.5 gm. doses to each rat as a supplement to the basal ration. In Curve I, Chart II, the result of this feeding test is presented. The curve represents the average growth of ten rats. The average weekly gain per rat was 6.8 gm.

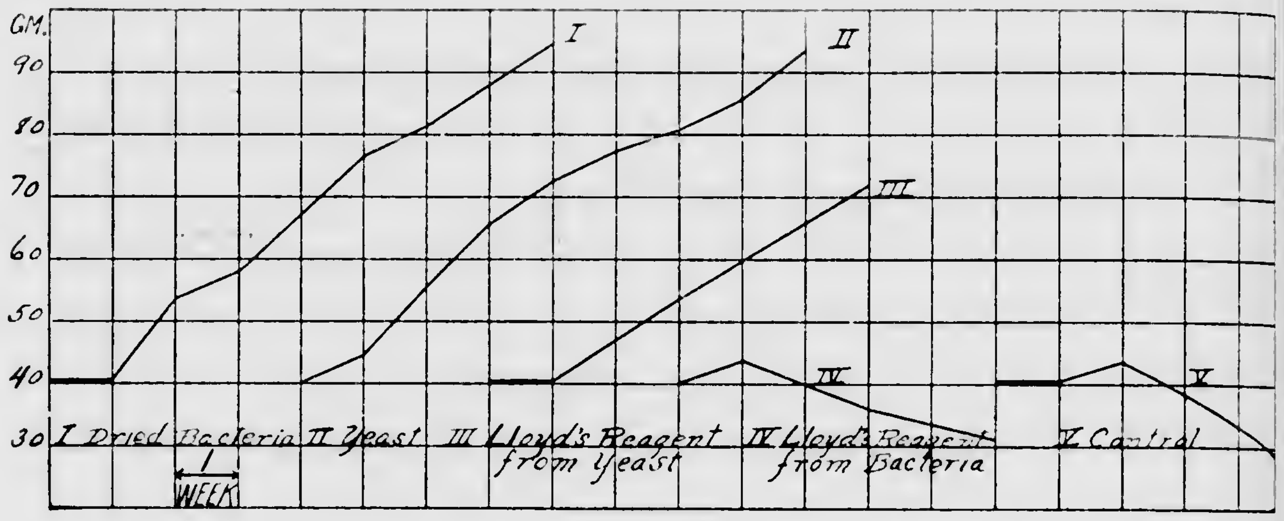

Chart II.

At the same time another group of rats, some of which were litter mates of those represented in Curve I, were fed the basal ration supplemented with 0.5 gm. of Fleischmann's special yeast. In Curve II the average growth of this group is presented. The average weekly gain per rat in this group was 6.4 gm.

There was some question as to whether the media (dehydrated nutrient agar) which were used in growing the large quantities of bacterial cells, did not carry appreciable amounts of the vitamin B complex. To test out this point, a liter of medium made from Bacto-Nutrient Agar was shaken with 10 gm. of Lloyd's reagent. The mixture was filtered and the filtrate evaporated to dryness. The residue was fed to rats in daily 0.5 gm. doses with the result as presented in Curve IV, Chart II. A similar test was made with autolyzed yeast, prepared as described by Seidell (11). Curve III represents the average growth of this group. A control group was also fed on the basal diet with the results portrayed in Curve V.

SUMMARY AND CONCLUSION.

Investigations were conducted on the fermented rumen contents of a Holstein cow, representative of a group of seventeen animals that were grown to maturity on a ration highly deficient in vitamin B complex. Alcoholic extracts of the fermented rumen contents were proved potent in the vitamin B complex through rat feeding trials.

One bacterium of the genus *Flavobacterium* was found about 90 per cent predominant in the rumen microflora. This organism was grown in large quantities on vitamin B-free media and fed to rats to the extent of about (12 per cent) of dried bacterial cells in a synthetic vitamin B-free ration. This test proved the bacterial cells to be highly potent in the vitamin B complex.

The results of this study warrant the conclusion that the vitamin B complex was produced in the rumen of our experimental cow by bacterial fermentation. This result offers a satisfactory explanation as to why cattle, unlike any other species of animal yet studied, have the ability to grow to maturity, to produce normal offspring, and to produce milk of normal dietary composition, on a ration that carries an insufficient amount of vitamin B complex to support growth and well being in rats.

Credit is due Dr. J. F. Shigley, of the Department of Animal Husbandry, for veterinary service in caring for the experimental heifer with the permanent fistula.

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THE ABSORPTION OF GLUCOSE FROM THE ALIMENTARY TRACT OF RATS DEPRIVED OF THE VITAMIN B COMPLEX[1] [2]

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The functional activity of the gastro-intestinal tract is dependent to some extent upon the presence of vitamins A and B in the diet. Cramer (1923) has found that the use of diets deficient in vitamin B caused atrophy of the lymphoid tissue of the ileum. In the absence of actively functioning lymphoid tissue, the assimilation and absorption of foods from the intestine is impaired. Cowgill (1926), Gross (1924), Plummer (1927), Nelson (1925) and McCarrison (1919) (1921) (1922) have found that vitamin B deprivation caused degenerative changes in the gastro-intestinal tract which prevented the normal functioning of the component parts. Several of the latter authors have observed that the alimentary rate decreased when diets deficient in vitamin B were fed to animals. At the same time there was a decrease in the tonus of the intestinal musculature. These several observations have led to the postulation of a decreased absorption from the intestine in the absence of vitamins A and B. Bergeim (1928) using his iron oxide method (1926) fed rats and guinea pigs rations deficient in vitamins A, B, C and D. He studied the digestive efficiency of normal animals and compared it with that of animals receiving inadequate diets. Little difference was observed between the ability of the two groups to digest starch, while the protein digestion values for the two groups did show some difference, being smaller for the groups receiving vitamin deficient diets. Bergeim does not believe "that these differences are great enough to support the view of an early or specific impairment of digestive function in these avitaminoses."

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[1] Published with the permission of the Director of the Agricultural Experiment Station as paper No. 463.

[2] The preliminary part of this investigation was carried on in the Department of Vital Economics at the University of Rochester and the latter part in the Department of Agricultural and Biological Chemistry at the Pennsylvania State College. Mr. Polansky assisted in the preliminary studies and Mr. Osgood in the later studies.

EXPERIMENTAL

The object of the present investigation was to determine whether or not the absence of the vitamin B complex in the diet of rats had any effect upon the absorption of glucose from the gastro-intestinal tract. Cori (1925) devised a method for studying the rate of absorption of various sugars from the intestinal tract of rats, and we have followed his technique, with some modifications. The principle of this method, as stated by Cori, is as follows: "A known amount of the substance under investigation is fed by stomach tube. After a given time the rats are killed and the amount of substance remaining in the intestines is determined quantitatively. The difference between the amount fed and the amount recovered from the whole intestinal tract is then the amount of substance absorbed."

A number of years ago Rubner (1902a) and more recently Kugler (1919) used a method for studying absorption from the alimentary tract of animals similar to that devised by Cori.

Our procedure differed from that of Cori in several ways. The rats used were older and on an average weighed more than those used by Cori. Solid food was withheld, but water was left at the disposal of the rats during the fasting periods. The normal Rochester rats were fasted for 48 hours before receiving the meal of glucose, but on account of the weakened condition of the rats on vitamin B-free diets, they were fasted for 24 hours only. Fasting periods of twenty-four hours were used with all of the other groups. With the exception of the Rochester rats, one hour absorption periods were used. Excellent checks were obtained in the determination of the amounts of glucose fed to the rats. The amount of sugar adhering to the outside of the catheter after its withdrawal was found to be negligible. The amounts of glucose remaining inside the catheter after the injection showed some variation and because of this the catheters were washed out each time after use, and the glucose in the wash water determined. The various catheters used retained different amounts of glucose. Several rats were killed with the stomach tube in place to confirm its location with the tip in the stomach. The amount of reducing substances present in the intestine of rats fasted for 24 hours was found to be small and was neglected in all calculations. Twelve animals were used in this particular phase of the invesitgation and in one case only was there more than a trace of reducing matter present. When rats had been fed sugar after a fasting period of 24 hours, no reducing substances were found in the cecum at the end of a one-hour absorption period. After having removed the alimentary tract it was cut open and washed carefully with successive portions of warm water until approximately 500 cc. of water had been used. The intestine after washing was placed in a volumetric flask and shaken with water, this being tested for the presence of glucose. No reducing substances were found to be present. Rats which were frightened and struggled when the stomach tube was being placed in position were not used. The sugar used in all of the experiments was C.P. d-glucose obtained from the J. T. Baker Company. The strength of the glucose solutions used ranged between 30 and 50 per cent, and the solutions were prepared 24 hours before using (we, of course, having accepted Cori's statement that concentration did not affect the rate of absorption). Bertrand's method was used for the quantitative determination of glucose.

None of the rats exhibited glycosuria during the absorption periods and there was evidence of diarrhoea once or twice only.

Before studying the effect of the absence of vitamin B on the absorption of glucose, a number of determinations were made using normal animals three to five months of age which had been on the colony ration (Mattill and Clayton, 1926) at Rochester. Later several groups of rats were placed on a vitamin B-free diet of the following composition: Casein (vitamin B-free) 18%, salt mixture (McCollum 185) 4%, corn starch 56%, lard 20%, cod liver oil 2%.

A record of the food intake of the Rochester group of animals on a vitamin B-free diet was not kept. These animals were placed on the deficient diet for 41 to 46 days, and at the end of this period they were in a greatly weakened condition. At the end of the diet period the rats were fasted for 24 hours, and the glucose absorption determined as in the case of normals using Cori's procedure.

In later work at the Pennsylvania State College two groups of rats were used, one group consisting of animals from the stock colony of pied rats and known as "Penn State" rats, and the other group consisting of animals obtained from the Albino Supply Company at Philadelphia, designated by the Company as "large size, toxicity" rats. The Penn State rats have proved to be very resistant to the effects of vitamin B deprivation and it has been suggested by associates of the writer at State College that a micro-organism capable of synthesizing vitamin B was a member of the intestinal flora, or that the intestinal flora was altered in some other way, so that toxic substances ordinarily formed and absorbed were absent. Wright (1921) has expressed the opinion that the explanation for the early decline in rats deprived of vitamin B lies in the fact that toxic products are formed in the abnormal length of time that food remains in the intestine. The toxins thus formed are absorbed and affect the general health of the animal. Fridericia and his coworkers (1927) have found that a number of their young rats, receiving vitamin B-free diets, grew, thrived and multiplied. The bacterial flora in the cecum of their rats differed from that of other rats, and the reaction of the contents of the cecum was acidic instead of neutral or alkaline.

It was owing to the fact that the Penn State rats did not show the effects of vitamin B deprivation that rats were obtained from another source. This second group of animals was kept in another building in order to avoid contact with the Penn State animals.

A record of the food intake was kept for all rats placed on vitamin B-free diets at the Pennsylvania State College. Because the original deficient ration tended to be lumpy and was easily scattered the composition was changed slightly at the latter institution. Instead of 56% corn starch, 66% was used and the percentage of lard reduced from 20 to 10. Otherwise the two rations were identical. The normal Penn State and the normal Albino Supply Company rats were kept on the diet used for the breeding stock (Dutcher, et al, 1925).

RESULTS

The results obtained with normal animals from the Rochester colony are shown in Table I. The average amount of glucose absorbed during the one- and two-hour periods was greater than that found by Cori, while the quantity absorbed during the third hour closely approximated his first results. (See also Chart 1.) Compared with those reported by Cori in two later papers (1928a) (1928b) our values for absorption during the one- and two-hour absorption periods are but slightly higher. Variations are to be noted in our results which are much greater than those found by Cori. For the one-hour absorption period the standard deviation calculated on the basis of absorption per 100 grams of body weight was 59.3 mgms. and the coefficient of variability 25.13% while on the basis of surface area the corresponding figures were 0.289 mgms. per sq. cm. and 20.60%. The standard deviation for the two-hour period on the basis of absorption per 100 grams body weight was 26 mgms. and the coefficient of variability 11.35% and the values obtained based on surface area were 0.107 mgms. per sq. cm. and 7.82%. For the three-hour period the standard deviation based on 100 grams body weight was 14.6 mgms. and the coefficient of variability 8.69%, while on the basis of surface area the standard deviation was 0.078 mgms. per sq. cm. and the coefficient of variability 7.85%. The results are more nearly uniform when the absorption periods are longer than one hour in length. The choice of litter mates in our work did not result in values which are any more nearly uniform.

The animals in the three groups show losses in weight during the 24 hours preceding the absorption period (Table I) which are somewhat lower than those observed by Cori.

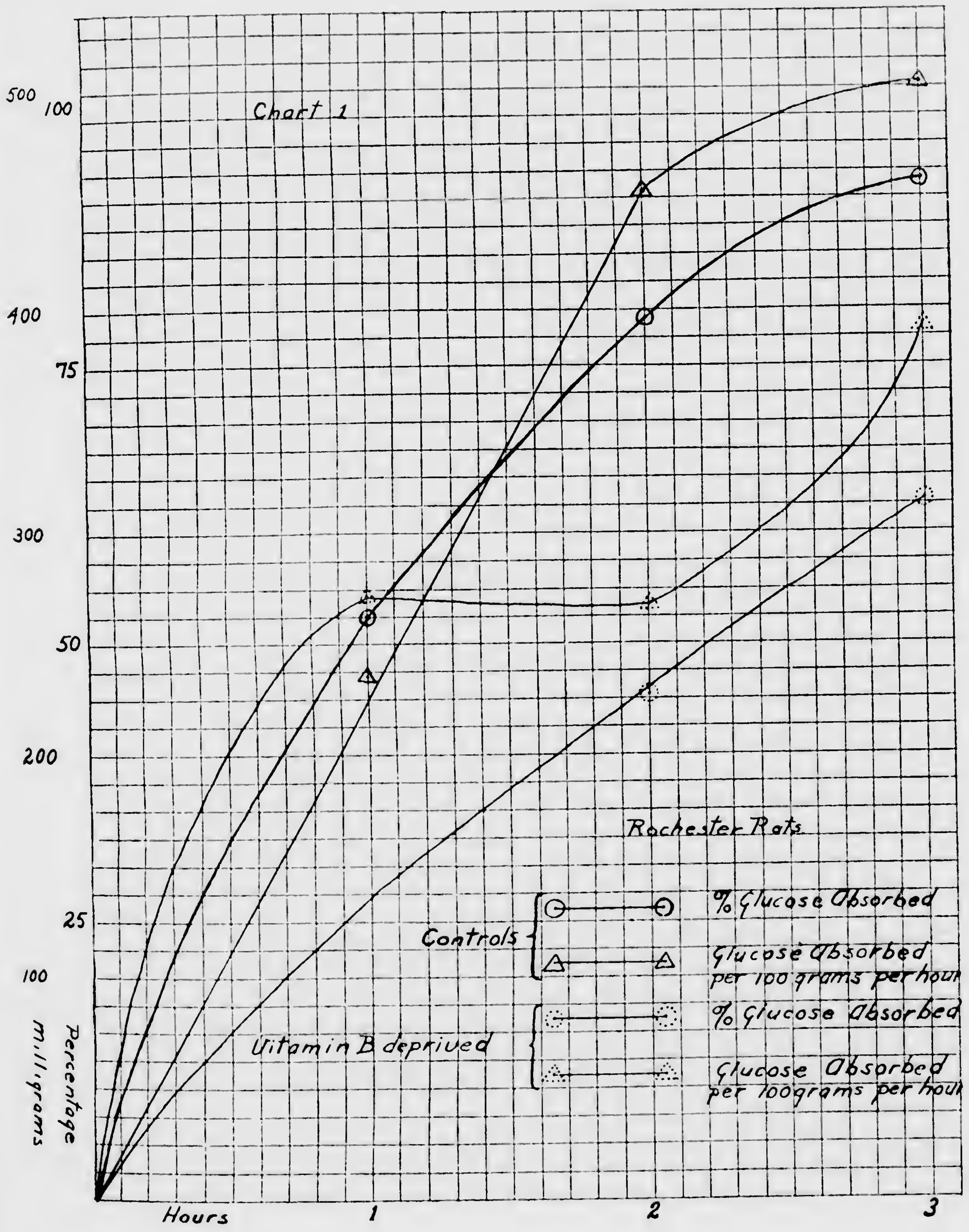

TABLE I. THE ABSORPTION OF GLUCOSE BY NORMAL ROCHESTER RATS[1]

Rat No. Males	Approx. Age mo.	Wt. before fasting gms.	Wt. after fasting 48 hrs. gms.	Wt. lost %	Glucose fed mgms.	Glucose Absorbed mgms.	Glucose Absorbed per hour per		Glucose Absorbed %
							100 gms. body wt. mgms.	Sq. cm. body surface mgms.	
					Absorption Period 1 hr.				
1	4	237	224	5.5	655	343	153	1.022	52.3
2	4	187	171	8.5	682	462	270	1.648	67.4
3	3	140	125	10.7	646	332	266	1.459	51.3
4	4	228	206	9.6	622	364	177	1.147	58.5
5	3-1/2	179	164	8.4	783	497	303	1.823	63.4
6	3-1/2	180	161	10.5	759	427	265	1.586	56.2
7	4	224	208	7.1	884	334	161	1.046	37.7
8	2-1/2	130	113	13.0	871	363	321	1.707	41.6
9	3-1/2	164	148	9.8	675	302	204	1.186	44.7
Average		185	169	8.2	731	381	236	1.40	52.6
Standard deviation							59.3	.289	
Coefficient of variability							25.13%	20.60%	

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[1] 35 per cent solution was given throughout.

TABLE I *cont.*

					Absorption Period 2 hrs.				
1	3-1/2	173	150	13.2	880	718	203	1.398	81.5
2	3-1/2	190	171	10.0	880	686	201	1.224	77.9
3	3-1/2	159	140	11.9	895	757	271	1.542	84.5
4	3-1/2	162	140	13.5	895	731	261	1.489	81.6
5	3-1/2	161	144	9.6	885	673	234	1.346	76.0
6	3-1/2	183	167	8.7	882	694	208	1.257	78.6
7	3-1/2	166	149	10.0	890	677	227	1.324	75.9
Average		171	152	11.0	887	705	229	1.37	79.4
Standard deviation							26	.107	
Coefficient of variability							11.35%	7.82%	
					Absorption Period 3 hrs.				
1	3-1/2	170	153	10.0	814	778	170	.996	95.5
2	3-1/2	180	163	9.4	762	745	152	.914	97.7
3	3-1/2	161	150	6.8	878	842	187	1.092	95.8
4	3-1/2	173	151	12.7	895	868	192	1.121	96.9
5	3-1/2	168	147	12.5	895	697	158	.917	77.8
6	3-1/2	178	160	9.1	877	781	163	.971	89.0
7	3-1/2	191	173	9.4	881	803	155	.948	91.1
Average		174	157	10.0	857	788	168	.994	91.9
Standard deviation							14.6	.078	
Coefficient of variability							8.69%	7.85%	

Slightly over 50% of the glucose injected was absorbed in one hour, just under 80% for two hours and just over 90% for three hours. When the amount of sugar absorbed per 100 grams of body weight was plotted against time, a straight line was obtained by Cori but is not obtained in our results (see Chart 1). Practically speaking a straight line is obtained for the first two hours, but during the third hour there is a marked falling off in the absorption rate. An absorption of 52.6% of the glucose during the first hour, leaves 47.4% remaining in the alimentary tract. If 52.6% of the remaining 47.4% were absorbed during the second hour, 24.9% would be taken up, making the total amount absorbed 77.5% as against the figure of 79.4% which was found. If 52.6% of the remaining 22.5% were absorbed during the third hour, 11.8% would be taken up making the total absorption 89.3% in place of 91.9% which is found. It would appear from this agreement that the amount of glucose absorbed at all times is a function of the concentration of glucose remaining in the alimentary tract. Calculation of Cori's results on a similar basis reveals that the absorption for the first hour was 30.7%, leaving 69.3% unabsorbed. If the same percentage of the remaining 69.3% were absorbed the second hour, 21.3% would be taken up, making a total absorption of 52% as compared with 45.2% actually found. Of the remaining 48% a further absorption of 30.7% in the third hour would give 14.6%, making the total amount of glucose absorbed 66.6% as compared with 69.4%, the value actually found. Although the absorption was at a slower rate, there appears to be the same dependence upon the residual sugar as in our own results, upon the basis of percentage absorption. Variations are found in Cori's work which approximate those which we have obtained.

Table II contains the results obtained with rats from the Rochester colony which had been placed on a diet containing no vitamin B for periods of 41 to 46 days. Here again marked variations are to be noted among the individual total amounts of glucose absorbed and in the amounts absorbed per 100 grams of body weight per hour. The standard deviation based upon the absorption per 100 grams body weight for the one-hour period was 77.7 mgms. and the coefficient of variability 28.67% while the corresponding results calculated on the basis of surface area were 0.410 mgm. per sq. cm. and 27.78%. For the two-hour absorption period the standard deviation based upon 100 grams body weight was 29.1 mgms. and the coefficient of variability 21.72% while the values obtained on the basis of surface area were 0.182 mgms. per sq. cm. and 23.39%. Larger amounts of glucose were absorbed per 100 grams of

TABLE II. THE ABSORPTION OF GLUCOSE BY ROCHESTER RATS ON A VITAMIN B-FREE DIET[1] [2]

Rat No. Males	Weight time of placing on diet gms.	Weight Time Killed gms.	Weight Lost %	Glucose Fed mgms.	Glucose Absorbed mgms.	Glucose Absorbed mgms. per hour per		Glucose Absorbed %
						100 gms. body wt.	sq. cm. body surface	
Absorption Period 1 hr.[3]								
1	255	142	44.3	1015	263	188	1.062	25.9
2	212	131	38.2	1209	257	196	1.095	21.2
3	190	119	37.3	1282	219	184	.995	17.1
4	186	103	44.6	1163	296	288	1.480	25.4
5	202	136	32.6	1287	433	319	1.799	33.6
6	188	114	39.3	1256	459	402	2.145	36.5
7	222	124	44.1	1255	397	320	1.759	31.6
Average	208	124	40.1	1209	332	271	1.476	27.3
Standard deviation						77.7	.410	
Coefficient of variability						28.67%	27.78%	
Absorption Period 2 hrs.[4]								
8	230	148	35.6	867	301	102	.569	34.7
9	243	154	36.6	870	502	163	.960	57.7
10	240	149	37.9	867	319	107	.623	36.7
11	260	157	39.7	956	507	162	.957	53.0
Average	243	152	37.5	890	407	134	.778	45.5
Standard deviation						29.1	.182	
Coefficient of variability						21.72%	23.39%	
Absorption Period 3 hrs.[4]								
12	228	137	39.9	871	535	130	.737	61.4
13	240	145	39.5	879	569	131	.755	64.7
Average	234	141	39.7	875	552	131	.746	63.1

[1] The approximate age of these rats at time of placing on diet was 4–1/2 months.
[2] The rats were fasted for 24 hrs. only.
[3] 50 per cent solution given.
[4] 35 per cent solution given.

body weight during the first hour than for normal rats. The reason for this is related to the higher percentage of glucose fed. See below.

The average weight of the rats after vitamin B deprivation for 41 to 46 days was 124 grams, although the original weights averaged higher than for the normal series. This smaller body weight would inevitably result in greater energy requirement per unit of weight, provided the vigor of the animals was kept up. It was of interest to see whether the absorption would be greater also per unit of body surface. Accordingly the absorption was calculated to milligrams per sq. cm. of body surface, using Meeh's formula ($S = kW^{2/3}$) in which the constant k has a value (Rubner (1902b)) of 9.1. Hill and Hill (1913) using results obtained with five rats, obtained an average value for k of 9.93, however we have used the value 9.1 for k in our calculations. The result indicates a closer relationship to body surface than to body weight during the one-hour absorption period (Tables I and II). There is a difference on this basis between the normal and vitamin B deprived animals of 5.71% while on the basis of body weight the difference is 15% during a one-hour absorption period. *If, however, the values obtained during the two-hour absorption period are used, there is a difference between the normal and vitamin B deprived animals on the basis of surface area of 76.09% while on the basis of body weight this difference is 55.97%.*

In starting the series on the vitamin B-free animals a change was made to a 50 per cent glucose solution with the thought that the greater concentration would reveal any defect of absorption all the more clearly, as indeed it does. But because such marked variations were being found, the percentage concentration of the glucose fed was reduced again during the two- and the three-hour absorption experiments to 35 per cent and the variations in the amounts of glucose absorbed were smaller. Notwithstanding the difference in concentration the percentages of glucose absorbed during the one- two- and three-hour periods all were materially less than those found for normal animals, as were the total weights of glucose absorbed.

The average results indicate a decreased glucose absorption during the second and third hours, there being a very marked decrease in the second hour, although the smaller amount fed leaves this conclusion a little in doubt. An absorption of 27.3% of the glucose during the first hour leaves 72.7% remaining. If 27.3% of the remaining 72.7% were absorbed the second hour, 19.8% would be taken up, making the total amount absorbed 47.1% as compared with 48.2% which was found. The same percentage of the remaining 52.9% would give 14.4% taken up, making the total absorption 61.5% in place of our result of 63.1%. It is evident also, from the curves in Chart 1, that absorption cannot be entirely independent of concentration.

The rate of absorption of glucose in successive hours was not studied in the investigation at the Pennsylvania State College, all absorption periods being of one hour's duration and the strength of solution given always 35%. Table III contains the results obtained for the glucose absorption by normal rats, a few of which were litter mates, of the Penn State colony. These rats were older and larger than those used by Cori. The amount of glucose absorbed per hour per 100 grams of body weight varies within rather wide limits and there appears to be no relationship between the amount of glucose fed and the amount absorbed. The standard deviation per 100 grams body weight was 46.64 mgms. and the coefficient of variability was 33.55%. The standard deviation based on 1 sq. cm. surface area was 0.315 mgms. and the coefficient of variability 33.31%. It is likely that the absorption would be more nearly uniform if the amounts of sugar fed were more nearly proportional to the body weight. The average percentage of glucose absorbed by this group of animals was 33.5 for a one-hour period, while the average found by Cori under similar conditions was 31.8%. Both of these values are much lower than those found for rats of the Rochester colony, due possibly to a difference in the ages of the rats used. Variations were of the same order of magnitude as those found by Cori.

The results shown in Table IV are those obtained in the study of glucose absorption from the intestine of rats which had been on the diet used for the animals receiving no vitamin B, with the exception that 0.25 grams of yeast were fed daily to each rat. Because more than one animal had to be kept in a cage, the food intake is an average value. The food intake of these animals was greater than that of the animals receiving a diet totally deficient in vitamin B. This group of animals lost an average of 21% of their weight during the feeding period, while the animals having a diet totally deficient in vitamin B lost on the average 32% of their weight during a period of similar length. The amount of glucose fed was nearly uniform for these rats and yet the amount of sugar absorbed varied markedly. The standard deviation for glucose absorbed per 100 grams body weight was 43.10 mgms. and the coefficient of variability 28.92%. Using the figures for the absorption per square centimeter body surface the standard deviation was 0.259 mgms. and the coefficient of variability 27.63%. The percentage of glucose absorbed was somewhat less than that found for normal animals, but the absolute amount per 100 grams body weight is 149 milligrams as compared with 139 (average) for

TABLE III. THE ABSORPTION OF GLUCOSE BY NORMAL PENN STATE RATS

Rat No.	Approx. Age mo.	Wt. before fasting gms.	Wt. after fasting 48 hrs. gms.	Wt. lost %	Glucose fed mgms.	Glucose Absorbed mgms.	Glucose Absorbed mgms. per hour per		Glucose Absorbed %
							100 gms. body wt.	sq. cm. body surface	
1	6		293		998	305	104	.759	30.7
2	6		236		998	313	132	.906	31.3
3	6		249		678	213	85	.591	31.3
4	6		239		681	221	92	.631	32.6
5	6		213		681	172	81	.530	25.3
6	12		300		1179	417	138	1.046	35.4
7	6		181		1164	457	218	1.570	39.2
8	7	206	199	3.4	1010	369	185	1.187	36.5
9	7	213	203	6.6	932	390	196	1.254	41.8
10	7	218	194	6.9	919	374	185	1.190	40.7
11	7	205		4.2	932	224	115	.735	24.0
Average		211	228	5.2	925	314	139	.945	33.5
Standard deviation							46.64	.315	
Coefficient of variability							33.55%	33.31%	

TABLE IV. THE ABSORPTION OF GLUCOSE BY PENN STATE RATS ON A VITAMIN B-DEFICIENT DIET PLUS 0.25 GM. YEAST per DAY

Rat No. %	Wt. Time Placing on Diet gms.	Weight When Killed gms.	Weight Lost %	Glucose fed mgms.	Glucose Absorbed mgms.	Glucose Absorbed mgms. per hour per		Glucose Absorbed %	Average Daily Food Intake gms.
						100 gms. body wt.	sq. cm. body surface		
1	245	178	27.3	1008	366	206	1.208	36.3	7.0
2	233	162	30.4	992	322	199	1.191	32.5	5.8
3	272	208	23.5	978	222	107	.695	22.7	6.3
4	268	209	22.0	970	225	107	.702	23.1	6.3
5	259	203	21.6	1022	383	189	1.217	37.4	7.8
6	261	222	14.9	1045	425	191	1.274	40.6	8.0
7	228	179	21.4	974	254	142	.879	26.0	7.0
8	276	194	29.7	962	151	78	.495	15.9	7.0
9	255	213	16.4	955	336	158	1.035	35.1	8.0
10	249	200	19.6	981	201	100	.646	20.4	6.6
11	202	149	26.2	986	244	164	.954	24.7	5.8
Average	250	192	21.4	988	284	149	.936	28.6	6.8
Standard deviation						43.10	.259		
Coefficient of variability						28.92%	27.63%		

TABLE V. THE ABSORPTION OF GLUCOSE BY PENN STATE RATS ON A VITAMIN B-FREE DIET

Rat No.	Wt. Time Placing on Diet gms.	Weight When Killed gms.	Weight Lost %	Glucose fed mgms.	Glucose Absorbed mgms.	Glucose Absorbed mgms. per hour per 100 gms. body wt.	Glucose Absorbed mgms. per hour per sq. cm. body surface	Glucose Absorbed %	Average Daily Food Intake gms.
1	195	165	15.4	587	297	180	1.085	50.5	
2	160	126	21.2	593	182	144	.796	30.6	
3	263	174	33.8	697	362	208	1.276	51.8	4.9
4	223	142	36.3	703	305	215	1.595	43.4	4.9
5	265	184	30.6	701	175	95	.594	25.5	4.9
6	250	165	34.0	703	325	197	1.187	46.2	4.7
7	291	207	28.5	698	181	88	.568	25.8	5.8
8	309	207	33.0	698	224	109	.703	31.7	5.8
9	317	237	25.2	696	258	109	.740	37.0	5.8
10	260	161	38.1	977	217	135	.806	22.2	4.8
11	260	173	33.5	963	180	104	.637	18.5	4.8
12	305	215	29.5	975	300	140	.919	31.8	
13	294	159	45.9	1056	344	216	1.288	32.5	
14	342	187	45.3	1041	326	174	1.095	31.3	6.9
Average	248	179	32.2	792	269	151	.949	34.1	5.33
Standard deviation						44.87	.299		
Coefficient of variability						29.72%	31.52%		

the normals. The results obtained with normal Penn State rats which had received a diet deficient in vitamin B for 33 to 39 days are given in Table V. The amount of glucose fed was not constant, but, as noted before, there was no relationship between the amount fed and the amount absorbed. Neither body weight nor loss in body weight appear to have any effect upon the amount of glucose absorbed. The rats in this group had lost an average of 32% of their body weight, while the rats obtained from the Albino Supply Company had lost 39% and the Rochester rats 40% of their body weight under similar conditions. The amount of glucose absorbed by the vitamin B deprived animals was less than that absorbed by the normal rats.

The absorption per 100 grams body weight per hour was slightly less with the normals than with the animals receiving the deficient diet; however the difference in these figures is less than 10% and the significance of this is to be questioned. Although the percentage absorption of glucose is somewhat less than normal in those animals which had been on a diet partially deficient in vitamin B, the percentage of absorption in case of the animals on a diet totally deficient in vitamin B is slightly greater than that of normals. This observation is of particular interest in light of the fact that the Penn State rats have been able to resist the effects of vitamin B deprivation. The other two groups of animals did show a decrease in the amount of glucose absorbed. The standard deviation calculated on the basis of absorption per 100 grams body weight was 44.87 mgms. and the coefficient of variability 29.72% while the standard deviation for the results based upon one sq. cm. surface area was 0.299 mgms. and the coefficient of variability 31.52%.

Table VI shows the values obtained for glucose absorption with normal animals purchased from the Albino Supply Company. The amounts of glucose fed were comparatively uniform and the amounts absorbed were also more nearly uniform. The standard deviation based upon absorption per 100 grams body weight was 17.06 mgms. and the coefficient of variability 12.83%, while for absorption per square centimeter body surface the standard deviation was 0.111 and the coefficient of variability 14.87%. Body weight does not appear to influence the total amount of glucose taken up. However, the heavier rats tend to absorb less glucose per 100 grams of body weight than do the lighter animals. The amount of glucose absorbed per 100 grams of body weight per hour is considerably less than the amount observed by Cori. A difference in age of between four and eight months had little effect upon glucose absorption. The percentage of absorption is slightly less with these rats than with the Penn State

Table VI. The Absorption of Glucose by Normal Albino Rats on an Adequate Diet

Rat No.	Approx. Age mo.	Wt. before fasting gms.	Wt. after fasting 24 hrs. gms.	Weight lost %	Glucose fed mgms.	Glucose Absorbed mgms.	Glucose Absorbed mgms. per hour per 100 gms. body wt.	sq. cm. body surface	Glucose Absorbed %
1	8	276	259	6.15	1033	333	128	.901	33.2
2	8	266	252	5.26	1006	295	117	.813	29.4
3	8	224	212	5.35	1012	230	109	.711	20.2
4	4	315	296	6.03	985	318	108	.787	32.3
5	4	327	304	7.03	952	257	84	.625	26.9
6	4	326	304	6.74	942	267	88	.649	28.2
7	4	285	270	5.26	944	334	124	.879	35.4
8	8	254	246	3.15	973	283	115	.792	29.9
9	4	335	322	3.88	973	250	78	.587	25.9
Average		290	274	5.42	980	285	106	.749	29.04
Standard deviation							17.06	.111	
Coefficient of variability							16.10%	14.87%	

normals, and materially less than with normals from the Rochester colony.

The results obtained with the rats that came from the Albino Supply Company, which had been on a diet partially deficient in vitamin B, are shown in Table VII. The amount of glucose fed to this group of animals has been fairly uniform. These rats had lost on an average 25% of their original body weight during the period of the diet as compared with a 21% loss found with the Penn State rats. The food intake of these rats was greater than with the corresponding group on a diet totally deficient in vitamin B and somewhat higher than in the case of the Penn State rats on the same diet. The amount of glucose absorbed varied within wide limits. The standard deviation calculated on the basis of absorption per 100 grams of body weight was 30.6 mgms. and the coefficient of variability 23.91%, while the corresponding figures for absorption per square centimeter body surface were 0.196 mgms. and 23.42%. There is no apparent correlation between the body weights of the rats and the total amounts of glucose absorbed. The amount of glucose absorbed is slightly less than that observed for the normals of this group. The percentage of glucose absorbed is a fraction of a per cent higher than that of the control group.

Table VIII contains the results obtained with Albino rats which had been on a diet totally deficient in vitamin B for a period of from 39 to 45 days. The amounts of glucose fed were nearly uniform and closely approximate the amounts fed to the corresponding group of rats on a diet partially deficient in vitamin B. These rats had lost an average of 39% of their body weight during the feeding period as compared with an average loss of 32% found for the Penn State rats, and 40% for the Rochester rats under similar conditions. The amounts of glucose absorbed were not at all constant nor were the amounts absorbed per 100 grams body weight per hour. The standard deviation based upon the absorption per 100 grams body weight was 34.1 mgms. and the coefficient of variability 33.46%, while for absorption per square centimeter body surface the standard deviation was 0.204 mgms. and the coefficient of variability 32.48%. The total amount of glucose absorbed was materially less than with the normal animals and those on a vitamin B-deficient diet plus yeast. The absorption per 100 grams body weight was slightly less than with the control rats. However, the percentage of glucose absorbed was about one-third less. The average of 20% absorption is the lowest value found for any of the rats on experimental diets. There was no correlation between body weight and the total amount of glucose absorbed. The food intake of the individual animals has had no apparent effect upon the amount of absorption,

TABLE VII. THE ABSORPTION OF GLUCOSE BY ALBINO RATS ON A VITAMIN B-DEFICIENT DIET, PLUS 0.25 GM. YEAST PER DAY

Rat No.	Wt. Time of Placing on Diet gms.	Wt. When Killed gms.	Wt. Lost %	Glucose fed mgms.	Glucose Absorbed mgms.	Glucose Absorbed mgms. per hour per		Glucose Absorbed %
						100 gms. body wt.	sq. cm. body surface	
1	288	217	24.6	792	235	108	.715	29.7
2	302	235	22.1	792	180	77	.519	22.8
3	278	218	21.5	780	195	90	.592	25.0
4	289	220	24.0	844	280	127	.844	33.1
5	287	218	24.0	923	282	129	.856	30.5
6	259	203	21.6	915	257	127	.818	28.1
7	294	232	21.0	891	189	82	.550	21.3
8	302	230	23.8	990	288	125	.843	29.3
9	299	211	29.4	981	327	155	1.014	33.3
10	273	198	27.4	959	302	153	.977	31.5
11	285	180	36.8	956	249	138	.858	26.1
12	290	200	31.0	970	356	178	1.144	35.6
13	301	233	22.5	964	394	169	1.143	40.8
Average	288	215	25.3	904	273	128	.836	29.7
Standard deviation						30.6	.196	
Coefficient of variability						23.91%	23.42%	

TABLE VIII. THE ABSORPTION OF GLUCOSE BY ALBINO RATS ON A VITAMIN B-FREE DIET

Rat No. Males	Wt. Time of Placing on Diet gms.	Weight When Killed gms.	Weight Lost %	Glucose fed mgms.	Glucose Absorbed mgms.	Glucose Absorbed mgms. per hour per		Glucose Absorbed %	Average Daily Food Intake gms.
						100 gms. body wt.	sq. cm. body surface		
1	313	202	35.4	828	236	117	.753	28.4	6.6
2	276	166	39.8	897	167	101	.608	18.6	6.4
3	306	186	39.2	891	192	103	.648	21.4	5.6
4	292	185	36.6	897	213	115	.721	23.8	5.6
5	294	178	39.4	1006	167	94	.580	16.6	5.9
6	277	149	46.2	974	112	75	.438	11.4	7.5
7	300	179	40.3	897	225	125	.778	25.0	6.2
8	292	166	43.1	897	218	131	.793	24.3	6.0
9	329	205	37.6	897	180	88	.569	20.0	6.0
10	303	181	40.2	897	60	33	.206	7.0	6.0
11	298	179	39.9	990	277	155	.958	27.8	6.0
12	277	150	45.8	944	223	149	.868	23.6	5.2
13	301	195	35.2	799	94	48	.307	11.9	7.0
14	270	165	38.8	830	213	129	.778	25.6	7.0
15	305	198	35.0	830	130	66	.421	15.7	7.0
Average	296	186	39.2	898	181	102	.628	20.0	6.26
Standard deviation						34.1	.204		
Coefficient of variability						33.46%	32.48%		

although it should be mentioned that group feeding was used because of lack of available space, and two or three rats were kept in a cage.

Discussion of Results

Mattill (1923) observed that rats deprived of vitamin B which have received either glucose or sucrose show a rise in the respiratory quotient, although this rise is less than in the case of normals. He has suggested that the reason for this may lie in a delayed absorption. Our results tend to show that this may be true (See Chart 1). Miller and his coworkers (1920) have observed, however, with human subjects, that feeding large amounts of glucose or sucrose in concentrated solution depressed gastric secretion and delayed evacuation of the stomach. Calculated on the basis of grams of sugar per kilogram of body weight the amount of sugar used for humans by Miller and the amount used by us for rats is approximately the same.

Experiments of this kind may be vitiated by the fact that refusal of food accompanies vitamin B deprivation as Mitchell (1927) (1928) has pointed out. Luchetti (1918) has found that absorption from the intestine is slower in dogs which have fasted than in normal dogs. It is extremely difficult to distinguish between the effects of vitamin B deprivation and inanition.

The question may be raised as to possible explanations for the difference in behavior between the Penn State rats and the rats of the other two groups. Young rats of the Penn State colony show little or no effect of vitamin B deprivation over a period of a year other than a failure to grow. Older rats of this same colony when placed on a diet containing no vitamin B lose weight slowly and their appetite diminishes. It should be remembered that the food intake of the Penn State rats remained at a higher level during vitamin B deprivation than that noted for rats obtained from the Albino Supply Company. The Penn State rats did not lose as much weight as rats of the other groups under the same conditions. The Penn State rats were pied, and the other groups were albino, so that racial differences may have affected the results. With rats of the other two groups there was a marked decrease in the absorption of glucose when vitamin B was absent from the diet. We cannot account for the lowered absorption with the Penn State rats that had been receiving yeast with the partially B-deficient diet. Additional observations might have shown this difference to be of a lesser value.

The variations in the percentage glucose absorbed with the Penn State rats on a vitamin B-free diet were much greater than those noted with the two other groups which makes the interpretation of these results difficult. With the Penn State rats it would appear that vitamin B deprivation had variable effects. The differences noted with animals of different origin illustrate the necessity of using a standard group of animals for such investigations. The experimental work with the Rochester rats was performed during the winter months, while the work with the other two groups of rats was done during the spring and summer. That the time of year during which the observations were made may account for some of the differences noted between the Rochester rats and the other groups is to be doubted because all were subjected to about the same temperature, although Riddle and Honeywell (1924) have found that cold weather causes a marked lowering in the concentration of blood sugar in pigeons kept outdoors.

Mottram, Cramer and Drew (1922) have shown that the presence of vitamins in the food, particularly vitamin B, causes a more rapid absorption of fat from the intestine. Never (1928), after having studied the effect of vitamin-free diets upon the secretion and motility of the intestinal tract, and upon appetite, concludes that these three are disturbed secondarily and the most significant disturbance lies in absorption from the intestinal tract.

Seth and Luck (1925) studied the absorption of amino acids from the intestinal tract by analyzing the blood of the animals used (rabbits and dogs), determining the amino acid and urea content. In general there was a rapid rise in the amino acid nitrogen in rabbit's blood during the first two hours, following the ingestion of amino acids, after which there was a smaller increase or a decrease. In a subsequent experiment, in which dogs were used, the amino acids were injected into the small intestine by means of a hypodermic needle and a pipette. The amino nitrogen in the arterial blood tended to increase rapidly after the injection, and gradually fell off, the same holding true for the amino nitrogen in the portal blood. The urea content of the blood increased more rapidly early in the period and tended to decrease after one or two hours. The results indicate that absorption does not take place at a uniform rate, for if it did, we should expect to find a consistent and uniform increase in the amount of amino nitrogen in the portal blood.

The statistical treatment of the results obtained is of questionable value, because of the small numbers of animals used in some of the groups, except to emphasize the large variability of the results.

The authors plan to continue this investigation.

SUMMARY

1. The method devised by Cori for the quantitative study of the absorption of glucose by the whole alimentary tract has been used.

2. Three different groups of animals were used, one group from the Rochester colony, a second from the Penn State colony and a third from the Albino Supply Company. The Penn State rats were pied, while the other two groups were albino. The results obtained with the different groups do not correspond closely.

3. The rate of absorption of glucose was not uniform for the one- two- and three-hour periods, this being particularly noticeable in case of the rats which had received a diet deficient in vitamin B.

4. The total amount of glucose absorbed and the amount absorbed per 100 grams of body weight per hour by normal and vitamin B-deprived animals varied within wide limits. These variations were not dependent upon body weight nor upon the actual amount of glucose fed.

5. Absorption per square centimeter of surface area varies markedly, although the results indicate that there is a slightly closer relationship between absorption and body surface than between absorption and body weight.

6. The percentage of glucose absorbed during one-, two- and three-hour periods appeared to be dependent upon the amount of glucose remaining unabsorbed in the alimentary tract.

7. The Rochester rats which had been on a diet containing no vitamin B absorbed a smaller percentage of the glucose fed than did normal animals.

8. Vitamin B deprivation had little effect upon absorption in rats of the Penn State colony, rats on a diet totally deficient in vitamin B absorbing practically the same amount of glucose as the normal animals. Rats which had been on a B-deficient diet but had received yeast did absorb a lesser percentage of the glucose fed.

9. Rats from the Albino Supply Company, which had received a diet containing no vitamin B absorbed a smaller amount of the glucose fed than did normal animals. Rats on the same B-free diet which had received yeast showed little difference in absorption as compared with normals.

10. The percentage of glucose absorbed by normal animals of the Penn State colony and the Albino Supply Company closely approximates that found by Cori.

11. The variation in the percentage of absorption with our normal animals is somewhat greater than the percentage variation to be noted between the absorption coefficients found by Cori.

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AN ADAPTATION OF BERGEIM'S FECAL INDOLE METHOD FOR THE QUANTITATIVE DETERMINATION OF INDOLE IN BACTERIAL CULTURES.

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During the course of a recent investigation, one of the writers (P.) desired to determine quantitatively the amount of indole present in bacterial cultures. Prior to this time, Bergeim's method (1) had been found to be satisfactory for the estimation of indole in feces.

Bergeim believed that of the large number of color reactions which had been used for the determination of indole, only the β-naphthoquinone sodium monosulfonate reaction of Herter and Foster (2, 3) offered much promise as a basis for the accurate determination of indole in feces. On the basis of this experience we have investigated the applicability of this method for the quantitative determination of indole in bacterial cultures.

Bergeim's method consists of a steam distillation of a feces suspension in an alkaline medium to remove phenols. The distillate, which contains indole and ammonia, is redistilled from an acid solution or treated with permutit (exchange silicate) to remove ammonia. A portion of the ammonia-free distillate is treated with β-naphthoquinone sodium monosulfonate, the blue indole compound formed being extracted with chloroform and determined colorimetrically.

A survey was made of other methods which had been recommended for the determination of indole quantitatively in bacterial cultures. Fellers and Clough (4) made a comprehensive study of this problem several years ago, and discussed the relative merits of the various methods which had been used prior to the time their work was undertaken. They have classed the Herter and Foster method as "fairly satisfactory" but they do not recommend Bergeim's modification of this procedure for accurate work. They concede that the method is useful for separating indole quantitatively from skatole but they say that "the procedure is involved and the reagent exceedingly difficult to obtain." Another objection was that the reagent did not keep well on standing. After studying a number of procedures, Fellers and Clough decided to use the Ehrlich (5) *p*-dimethylaminobenzaldehyde reaction for their determination of indole.

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* Mr. Kilborn assisted in the later studies made at Pennsylvania State College.

Zoller (6) has objected to the use of Bergeim's method because (*a*) indole had to be separated from phenols and ammonia and this procedure was involved, (*b*) the reagent deteriorated rapidly and was difficult to obtain, and (*c*) a colorimeter had to be available for the determination.

The reagent may be obtained from the Eastman Kodak Company at present, the price of 10 gm. being 90 cents. This amount of β-naphthoquinone sodium monosulfonate is sufficient for approximately 500 determinations, and, if the solution containing this reagent is kept in the ice box, it does not deteriorate during a period of 3 days. When a precipitate forms, the reagent should be discarded. The separation of indole from phenols requires distillation from an alkaline medium and the subsequent removal of ammonia by the use of permutit or a second distillation from an acid solution. This procedure does require time, but it is not at all difficult to carry out with average equipment, and a colorimeter is usually available in modern laboratories.

EXPERIMENTAL.

Before Bergeim's method was used to determine the amount of indole present in bacterial cultures, a study was made of the percentage of indole recovered from water solutions, sterile 2 per cent solutions of Witte's peptone in water, and bacterial cultures in peptone water, to which definite amounts of a standard indole solution had been added just prior to the distillation. The bacterial cultures were prepared by inoculating 100 cc. of peptone water with 0.2 cc. of a feces suspension prepared according to Torrey (7), the indole being determined after 48 and 96 hours incubation periods. A definite amount of the culture medium, usually 10 cc., was removed and diluted to 400 cc. before distilling. The water solutions of indole were prepared by adding known volumes of a standard indole solution to 400 cc. of water, while the peptone water solutions were prepared by diluting definite amounts of peptone water, usually 10 cc., to 400 cc. with water and adding known volumes of a standard indole solution. The solutions containing indole were distilled from 800 cc. Kjeldahl flasks which were attached to connecting bulbs leading to 24 inch Liebig condensers arranged in an upright position. Glass delivery tubes were fitted to the ends of the condensers so that the distillate passed directly into 50 cc. of water in the receivers which were 500 cc. volumetric flasks. Prior to the distillation process, 5 cc. of 10 per cent KOH and approximately 2 gm. of paraffin were added, the latter to prevent foaming. The amount of KOH added placed the pH in the range of 8.5 to 10.0, which Zoller (8) found to be optimum for the recovery of indole from solutions. The distillation was carried out by the combined use of a flame and steam. The steam, obtained from a high pressure line (about 50 pounds) was passed through the contents of the flask until the volume in the receiving container amounted to 500 cc. This insured a maximum recovery of the indole which had been added to the solutions.

The initial heating must be carried on with caution to avoid excessive foaming and loss of indole by a too rapid volatilization. Although the larger amount of indole passes over with the first 100 cc. of the distillate, as Fellers and Clough have pointed out, it has been observed by the writers that some indole remains unvolatilized and the method loses its quantitative aspect unless approximately 500 cc. of the distillate are collected and the volume of the contents of the Kjeldahl flask is reduced to about 150 cc. Water is added to the solution by the condensation of steam, and if the flow of steam is too rapid, it is impossible to reduce the final volume to 150 cc. The steam flow may be controlled by regulating outlet valves on the steam line.

50 or 100 cc. of the distillate, depending upon the amount

of indole present, were transferred to a 500 cc. Erlenmeyer flask and 20 gm. of washed permutit[1] were added. The flask and contents were rotated moderately for 10 minutes, then, as soon as the permutit settled, the liquid was poured into a 150 cc. pear-shaped separatory funnel. The flask was rinsed with 5 to 10 cc. of water twice, the rinse water being decanted and added to the contents of the separatory funnel. 1 cc. of a 2 per cent solution of β-naphthoquinone sodium monosulfonate and 2 cc. of 10 per cent KOH were added, the contents of the funnel shaken, and the mixture allowed to stand for 15 minutes. A greenish-blue color developed and this colored compound was extracted first with a 10 cc. portion of chloroform and again with a 7 cc. portion. The chloroform extract was drawn off each time into a 15 cc. graduated cylinder. The amount of solvent used was sufficient to yield a final volume of 15 cc. The standard was prepared similarly and at the same time as the unknown by using 1 cc. of an indole solution containing 0.1 or 0.2 mg., diluted to 100 cc. The standard was usually set at 30 mm. in the colorimeter.

TABLE I.

Indole Recovery from Water Solutions, Peptone Water, and Bacterial Cultures in Peptone Water.

Series No.	Medium.	Indole added.	Average indole recovered.	No. of determinations.
		mg.	*per cent*	
1	Water solutions.	0.2–2.0	94.62	40
2	" " distilled with steam.	5.0	96.92	34
3	Water solutions distilled without steam.	5.0	88.58	18
4	2 per cent Witte's peptone water.	0.5–10.0	90.82	33
5	Bacterial cultures in 2 per cent Witte's peptone water.	0.5–5.0	90.85	28

Table I shows the results obtained during a study of the recovery of various amounts of indole from water solutions, peptone water, and bacterial cultures in peptone water to which definite amounts of indole had been added after incubation.

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[1] The permutit is washed to remove very fine particles which would otherwise cause a cloudiness in the solution to be used for the colorimetric comparison. The permutit may be recovered.

The average percentage of indole recovered from water solutions of indole in Series 1 was 94.62 per cent. The results in Series 2 and 3 illustrate the importance of using steam throughout the distillation period. The percentage of indole recovered by using steam continuously during the distillation was 96.92, while that recovered without the use of steam was 88.58. This result was to be expected, for substances relatively insoluble in water and possessing an appreciable vapor pressure at 100° are removed more efficiently from solution by steam distillation. The determinations made in Series 4 and 5 show the percentage of added indole recovered from sterile peptone water and bacterial cultures in peptone water. Approximately 91 per cent of the indole which had been added was recovered. Table II shows in detail the recovery of indole from bacterial cultures to which known amounts of indole had been added just prior to distillation. There appears to be a retention of indole by the peptone water. Fellers and Clough have observed this same phenomenon in the case of experiments in which attempts were made to remove indole from decomposing protein matter.

TABLE II.

Indole Recovered from Bacterial Cultures.

Indole in culture.	Indole added.	Indole found.	Indole recovered.
mg.	*mg.*	*mg.*	*per cent*
10.53	0.5	11.05	104.0
7.61	0.5	8.03	84.0
10.05	1.0	10.94	89.0
11.80	1.0	12.71	91.0
2.99	1.0	3.95	96.0
1.66	2.0	3.51	92.5
9.30	2.0	11.06	88.0
10.58	3.0	13.29	90.3
10.98	3.0	13.75	92.3
10.84	3.0	13.49	85.0
8.06	5.0	12.65	91.8
12.42	5.0	16.99	91.4
13.20	5.0	17.81	92.2
14.34	5.0	18.97	92.6

Attention has been called to the interference of the pale yellow color of the naphthoquinone reagent which modifies the pink color of the chloroform extract, but according to Bergeim this is scarcely appreciable when as much as 0.1 mg. of indole is present. With smaller quantities of indole this factor cannot be neglected. The standard used should contain approximately the same amount of indole as the unknown. Chloroform tends to form an emulsion during the extraction of the colored indole compound. The emulsion breaks within 1 or 2 minutes. It was observed that chloroform containing a trace of water did not tend to emulsify as readily as chloroform which contained no water.

The standard indole solutions should be freshly prepared, although, if kept in the ice box, little or no change takes place during periods of 3 or 4 days.

The color fades but slowly from the chloroform extracts, there being no appreciable change during a 20 minute period following the extraction.

Permutit (exchange silicate) proved to be entirely satisfactory for the removal of ammonia from the distillate.

Bergeim found indole to be removed quantitatively from solution by his process of distillation. We have found that an average of 90 per cent of the indole is removed from bacterial cultures under the conditions enumerated. Although the method is not 100 per cent quantitative, it is more nearly so than other methods which have been devised.

SUMMARY.

1. Bergeim's method for the determination of fecal indole has been adapted to the quantitative estimation of indole in bacterial cultures.

2. An average of 91 per cent of the indole added to peptone water or to bacterial cultures in peptone water has been recovered by use of this method.

3. The average percentage of indole recovered from water solutions ranged between 94 and 97.

4. Steam should be passed through the contents of the distilling flask during the entire period of distillation and the solution remaining in the flask at the end of the period should amount to approximately 150 cc.

5. The method outlined in the preceding discussion is more nearly quantitative than the method of Fellers and Clough, and the procedure, once the technique is standardized, is not involved.

6. The reagents required are comparatively inexpensive and may be obtained without difficulty.

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The Bactericidal Action of Indole.

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Gordon and McCleod[1] have called attention to the fact that there is little or no information to be found in the literature dealing with the antiseptic potency of indole. By experimentation these authors found indole to have an antiseptic effect ranging from 2 to 10 times that of carbolic acid, the efficiency depending upon the kind of bacteria exposed to its action. *B. coli* was one of the most resistant organisms but failed to grow in the presence of 0.1% indole. Gordon and McCleod believe that indole production may well be the mechanism by which *B. coli* maintains its dominant position in the fecal flora. During a study of the production of indole by fecal bacteria, information was desired concerning the effect of indole upon the growth of bacteria.

The medium used for the tests was 2% Witte's peptone water which had been adjusted to yield a pH of 7.6 after autoclaving. A solution of indole 1-1000 was prepared and filtered by pressure through a Seitz filter. Various amounts of this solution were added to 50 cc. portions of sterile peptone water. The flasks of medium were then inoculated with the organism to be studied by transferring a loopful of a 24-hour culture in 2% peptone water. All of the test cultures were incubated at 37° C. for 24 hours after which counts were made by the plating method.

The results obtained are shown in Table I. The growth of all of the microorganisms studied was hindered by the presence of indole in the culture medium. *Escherichia coli* was the most resistant of the organisms studied. Nevertheless its growth was prevented in the presence of 0.1% indole. *Salmonella paratyphi* was apparently very susceptible to the action of indole. Each result shown in the table was obtained by the analysis of separate cultures.

TABLE I.
Effect of Various Concentrations of Indole on Bacterial Growth.

Organism	Conc. of Indole	Total Count	Organism	Conc. of Indole	Total Count
		per cc.			per cc.
Escherichia coli	1-1000	0	*Aerobacter aerogenes*	1-1000	0
	1-1000	0		1-1000	0
	1-2000	0		1-2000	8,000
	1-2000	580,000		1-2000	4,000
	1-4000	63,000,000		1-4000	95,000,000
	1-4000	64,000,000		1-4000	83,000,000
	1-4000	39,000,000		1-4000	80,000,000
	1-4000	45,000,000		1-8000	450,000,000
	1-4000	60,000,000		1-8000	560,000,000
	1-4000	70,000,000		no indole	600,000,000
	1-8000	250,000,000		no indole	900,000,000
	no indole	370,000,000			
	" "	480,000,000			
	" "	500,000,000			
	" "	520,000,000			
Eberthella typhi	1-1000	0	*Salmonella paratyphi*	1-1000	0
	1-2000	500		1-1000	0
	1-2000	194		1-2000	22
	1-4000	40,000,000		1-2000	0
	no indole	207,000,000		1-2000	195
	no indole	230,000,000		1-4000	42,000,000*
				1-4000	0
				1-8000	270,000,000
				1-8000	210,000,000
				no indole	430,000,000
				no indole	810,000,000
Salmonella schotmulleri	1-1000	2	*Salmonella enteritidis*	1-1000	0
	1-2000	39,000		1-1000	0
	1-4000	87,000,000		1-2000	18,300
	1-4000	74,000,000		1-2000	21,000
	1-4000	80,000,000		1-4000	90,000,000
	no indole	480,000,000		1-4000	98,000,000
	no indole	550,000,000		1-4000	130,000,000
				no indole	300,000,000
				no indole	380,000,000

*Estimated.

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[1] Gordon, J., and McCleod, J. W., *J. Path. and Bact.*, 1926, **xxix**, 13.

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1. THE CHEMICAL APPROACH TO THE STUDY OF PROBLEMS OF TOBACCO FERTILIZATION[1]

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Of the many factors involved in the production of good-quality cigar-leaf tobacco, the potassium requirement is one of the most important. In order that we may understand more clearly its importance, let us first consider several well-established facts of a chemical or biochemical nature which bear directly or indirectly on this subject, and, furthermore, let us formulate a chemical interpretation for a number of otherwise unexplainable observations which have been noted by a number of workers in their investigations dealing with the relationship of potassium to the plant itself and to the quality of the product.

A knowledge of the condition of potassium as it normally occurs in the soil is of fundamental importance in a study of this kind. A small quantity of this element is usually present in either chemical or physical union with the soil organic matter. A portion of this potassium is readily available for plant growth, but complete availability can only be attained by decomposition of the organic matter. It is necessary, therefore, to pay close attention to the biological conditions of the soil and especially to those factors affecting the number and activities of the organisms which decompose organic matter. Work of the Cornell and other experiment stations has shown that the nutrition of these organisms must be provided for even before that of higher plants. The fact that additions of organic matter lead to a temporary depletion of soil nitrogen certainly emphasizes this point. What is true for nitrogen probably is true for other essential plant nutrients, although the effect may not be so apparent. With this in mind, we should profit from the results of investigations of the factors involved in the depletion of soil nutrients and especially with the factor of crop rotation. Moreover, the preceding crop may have a direct or indirect bearing on the activity of soil micro-organisms, apart from its effect on the available supplies of plant nutrients. There is, for instance, the favorable effect of clover as contrasted with the unfavorable effect of timothy, as shown by the work conducted at Cornell and elsewhere.

[1] Paper read as part of the symposium on "Tobacco Research" at the meeting of the Society held in Washington, D. C., November 23, 1928. Publication authorized by the Director of the Pennsylvania Agricultural Experiment Station as Technical Paper No. 473.

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The bulk of the soil potassium is held in inorganic combinations and is looked upon as being more or less unavailable for plant growth. Considerable quantities of this potassium may, nevertheless, become available for tobacco plants if the proper conditions are maintained. Especially is this true for that portion adsorbed by the inorganic soil colloids. Potassium so combined may be in equilibrium with the potassium dissolved in the soil solution, but the concentration of the latter is usually quite low, although an acid condition increases the amount going into solution. This quantity is directly available for plant nutrition, but for maximum absorption, however, the root-hairs must be in intimate contact with the individual soil particles so that the evolution of carbon dioxide from the plant may lead to the displacement of the adsorbed potassium, hydrogen taking its place in the colloidal complex. This form of potassium is quite satisfactory for nutritional purposes because no harmful acid radical is combined with it. Any factor, therefore, which favors root development will increase the amount of potassium absorbed by the growing plant which, on the whole, is desirable.

Some plants may absorb sufficient quantities of potassium from the soil to supply their needs, but this is not true for tobacco plants on account of their limited root systems. For this reason, therefore, it is necessary to have ample quantities of readily available potassium at their disposal to supply not only their nutritive requirements, but to provide sufficient quantities to insure good burning qualities. From this standpoint, therefore, the question of potash additions should receive careful consideration and in this connection it may be said that as far as work at the Pennsylvania Station is concerned muriate of potash should never be used as a fertilizer for cigar-leaf tobacco.

The addition of the common potash salts to soils relatively high in colloidal matter results in potassium being adsorbed and other adsorbed bases being replaced. The bases which are replaced subsequently combine with the acid radicals of the potash salts. Theoretically, it would appear possible for the acid radicals to be leached out of the soil. Our results show, however, that seasonal differences have no effect upon the quantity of chlorine absorbed by the plants from a given application of muriate of potash. On the other hand, these experiments have shown that sulfur is not absorbed very readily, or at least it does not accumulate in the leaves to any great extent.

Potassium in combination with acid radicals, such as the chloride and sulfate, will not function properly in combustion. Therefore, a wide ratio between potassium and such radicals is extremely important. For this reason, therefore, it would appear that the carbonate or nitrate of potash would be more desirable as a fertilizer than either the muriate or sulfate. The effects of carbonate of potash on the reaction of the soil and its physical condition is not desirable. Neither is the over-loading of the plant with readily available nitrogen compounds desirable. If, however, the conditions are such that the nitrogen of nitrate of potash is utilized efficiently by the plants, there results a liberation of potassium for its normal functions in growth and for catalytic activities during combustion.

In our work at the Tobacco Station located at Ephrata, Lancaster County, Pennsylvania, we have used 200 pounds of sulfate of potash per acre, believing that this amount was sufficient for our purpose. Analyses of the 1925 crop, however, showed a potash content of the leaves only slightly in excess of 2%. On the other hand, the lime content ran quite high, approximately 8%. In 1926 the potash and lime content were approximately 3% and 7%, respectively, while in 1927 each of these constituents approximated about 5%.

The quantity of potash in the leaves appeared to be directly proportional to the moisture conditions, while the lime content was inversely proportional. These results lead us to believe that a considerable amount of lime must have been leached out of the soil. Simultaneously a better root development of the plant was obtained resulting in more potash being absorbed. Considerable evidence is now available, however, which shows that the presence of relatively large quantities of active lime compounds in an ordinary soil seriously interferes with the solubility of the potash and its absorption by plants.

The burning qualities of the three crops closely paralleled their potassium content, and especially was this true as to the form in which this element occurred in the leaves. This brings out the question of the relation of potassium to the plant itself. It is thought that it plays a rôle in the synthesis of carbohydrates. In order that it may function in this capacity, however, it must be in a more reactive form than either the chloride or sulfate.

Another of the important rôles of potassium in plant metabolism is to neutralize the acids produced as a result of cell metabolism. Therefore, in order to function properly, it should likewise be in a more reactive form than either the muriate or sulfate. If a plant is properly supplied with potash, sufficient quantities are present for the neutralization of acids and to transport them to older tissues containing higher quantities of calcium where they may be precipitated. If these acids are not removed from the reactive system, the plant does not develop normally and is less resistant to the attacks of disease organisms. For this reason, any factor leading to an undue production of organic acids, such as heavy applications of nitrate nitrogen, should be avoided because it tends to increase the duties of the potassium present.

We believe that the more potassium found in the leaf in combination with organic acids, especially those containing hydroxy groups, the better the quality of the tobacco. Such tissues are usually of a low acidity which is conducive to good curing and fermentation. Moreover, it is a well-known fact that the catalytic effect of potassium during combustion is greater where it is combined with organic acids than if it were present as the muriate or sulfate.

The relation of potassium to other plant compounds should not be minimized. We recognize, for example, that iron may act as a catalyst during combustion if it is present in a certain form or forms. Moreover, it is also recognized that an alkaline reaction is more desirable for efficient combustion than is an acid reaction. Compare, therefore, the system of potassium chloride—iron chloride with the system potassium citrate—iron citrate. Under which condition would it be possible for both an alkaline reaction to be obtained during combustion and the subsequent precipitation of iron oxides? An additional point, however, must be taken into consideration. Where potassium salts of hydroxy organic acids are present in relatively large amounts, there is afforded an opportunity for iron to enter such a molecule to form a soluble complex. We believe that under such conditions, a better distribution of the iron occurs throughout the tissues. Certainly Hoffer's work shows this to be true. If iron does occur in this form it is precipitated during combustion in a colloidal state thus yielding a maximum surface for catalytic action. Moreover, it is found to possess magnetic properties when precipitated in this way, and as iron is supposed to show maximum catalytic powers when it approaches a mixture containing an equal quantity of FeO and Fe_2O_3, especially when an active basic compound is present, this form of iron and potassium is desirable.

In conclusion I wish to say that probably no agricultural plant presents such enormous possibilities for further and more detailed chemical studies than does the tobacco plant.

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PHYSICAL CHEMISTRY AT THE PENNSYLVANIA STATE COLLEGE*

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The catalog of The Pennsylvania State College lists as physical chemistry all courses which "involve both physics and chemistry or which lie on the border line between these subjects." The broad definition of the subject implied in these words seems to be both logical and workable. It emphasizes the essential unity of chemistry and physics and is in accord with the trend in both these subjects. The spirit of this definition carries through both the undergraduate and the graduate work. The result is that physical chemistry is primarily regarded as a jointly owned project of the physics, chemistry, and biochemistry departments. Graduate courses which contain a great deal of physics and some chemistry (such as atomic structure and spectroscopy) are taught as physical chemistry by the appropriate members of the physics department. Other graduate courses (such as catalysis) are taught as physical chemistry by members of the chemistry department who do no undergraduate teaching in physical chemistry. Still other graduate courses (such as x-rays, crystal structure, and colloids) are taught by those who give undergraduate instruction only in physical chemistry. Still other graduate courses (such as the physical chemistry of the cell) are given by members of the biochemistry department. In this way the joint control of the work by the physics and chemistry groups is emphasized without neglecting the "physical chemists."

Much the same situation exists in the undergraduate work. Lectures in classes in which the subject matter has a distinctly physical trend are given by members of the physics staff. Members of the chemistry staff give the lectures in classes in which the subject matter is predominantly chemical. Courses which emphasize strongly both the physical and the chemical viewpoints are taught by specialists whose training and experience has been in both subjects. In addition, two undergraduate courses in elementary physical chemistry are offered by the department of biochemistry which, for various reasons, is attached to the School of Agriculture rather than to the School of Chemistry and Physics. The will to coöperate is, however, so strong between those teaching physical chemistry in the two schools that little or no inconvenience seems to result.[1]

The problems of undergraduate instruction in physical chemistry at The

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* Contribution to the symposium on "The Teaching of Physical Chemistry," held by Divisions of Physical and Inorganic Chemistry and Chemical Education, at the 76th Meeting of the A. C. S., September 11, 1928, at Swampscott, Mass.

[1] For convenience in administration, the physical chemistry work of our School of Chemistry and Physics is listed as a division of the chemistry department.

Pennsylvania State College are rather unusual. Because of the probability that similar problems will come up in other colleges, it seems worthwhile to state in detail what these problems are and how they are being met. Four widely different types of undergraduate students are required to take courses in physical chemistry. These may be classified as follows:

(*a*) Students with weak mathematical training who are not required to take either physics or chemistry *as such* in their curriculum and whose lifework will probably not require a technical course in either science.

(*b*) Students with weak mathematical training who are required to take elementary courses in both physics and chemistry and who need elementary physical chemistry as part of their training.

(*c*) Students with at least one year of college physics, one and one-half years of college chemistry, and mathematics through calculus; whose major subject in college requires a good usable knowledge of at least a portion of physical chemistry.

(*d*) Students specializing in physics or chemistry. These men are required to take mathematics through differential equations.

These four groups present four different problems, not only because of their widely different preparation, but also because of their different viewpoints. It is not claimed that we, at Penn State, have found the best possible solution to the problem, but we believe we have at least a workable solution.

Group (*a*)—Certain of our college curricula, such as Arts and Letters, Commerce and Finance, and Education do not include even the elementary college courses in chemistry and physics. Most students in the curriculum in Education have no mathematics beyond what they had in high school and this does not have to exceed algebra up to quadratics and plane geometry. Students in the curricula of Arts and Letters and of Commerce and Finance are required as part of their general cultural training to take throughout the freshmen year a "survey course" in mathematics which covers in three hours per week the elements of algebra, trigonometry, analytic geometry, and calculus. All of these students are required by their respective schools to take also a one-year survey course involving both physics and chemistry as part of their cultural training. By definition, such a course, combining as it does simultaneous instruction in both physics and chemistry, is listed as physical chemistry. Those lectures which emphasize the physical side of the picture are given by a member of the physics staff; those which emphasize the chemical side are given by a member of the chemistry staff. By close coöperation of the two the lectures make a homogeneous whole. Demonstration experiments are carried on throughout the lectures, not by way of illustrating laws and rules given *ex cathedra* to the class by the lecturer, but rather as a means of providing definite facts on which to base a discussion from which the class discovers for itself the laws involved. This is one of the most popular courses in the college. It has about 460 students.

Group (*b*)—Students in the pre-medical and biochemical curricula are required to take the usual elementary courses in physics, and in inorganic, analytical, and organic chemistry. It is almost self-evident that these students need a good working knowledge of physical chemistry. Although they have mathematics through trigonometry, their whole mental bias is inclined to be non-mathematical. It is easy to say: "Put more mathematics in their curriculum," but it is hard to find the necessary time. It is easy to condemn a student for not liking mathematics, but it does not follow that only mathematical enthusiasts have a rightful place in the world, even though that world is constantly becoming more and more mathematical.

We seem to have our choice of two ways of solving the problem of teaching these men physical chemistry. We can give them merely the qualitative material that passed for physical chemistry twenty years ago, or we can try to give them a word-picture of what higher mathematics is really like, and then use the language and notation of calculus (with constant reference to the word-picture) in the effort to show these students how we arrive at modern physico-chemical ideas. At The Pennsylvania State College we have adopted the second of these alternatives. It is important in this connection to note that those who teach physical chemistry in the School of Agriculture and those who teach it in the School of Chemistry and Physics have arrived at practically the same solution of the problem. A conference of teachers from both schools shows that the work offered by one differs from that offered by the other mainly in minor details. We feel that the close parallelism between the courses developed independently by the two Schools is a strong argument in favor of the correctness of our solution of the problem. It will be of interest to describe the two courses more in detail.

All juniors in group (*b*) are required to take a full year of physical chemistry. Premedical students are scheduled in the same lectures and laboratory sections as men from groups (*c*) and (*d*). They are taught by men in the department of chemistry who are specialists in physical chemistry. Students in biochemistry and in certain agricultural curricula are scheduled in separate courses which are taught by a specialist in the department of bio-chemistry. It is taken for granted in both courses that the subject cannot be taught without the use of mathematics, but there is a constant effort in both courses to keep the mathematics to a minimum consistent with a proper understanding of physical chemistry. Whenever there is a choice between an elegant mathematical derivation and a word picture of a physical idea, the word picture is used. If a mathematical discussion is necessary for a proper understanding of a given topic, it is preceded by a qualitative word-picture, and the mathematical treatment is correlated step by step with the word picture. Although of necessity derivations of equations are given frequently in the lectures, every effort is made to keep from taking for granted any previous knowledge of mathematics. Even simple algebraic steps are explained in detail. This is in accord with the common experience that the average student does not carry over into one classroom all that he has learned in some other classroom. Nor is it to be expected that he will. If he knew enough about mathematics, physics, and chemistry to be able to correlate them himself during a lecture in physical chemistry, he would already know so much physical chemistry that he would not need to take the course. It is a common mistake in teaching to assume that correlations which are almost self-evident to the teacher will occur at all to the unaided student.

Not only are the bio-chemistry and the chemistry departments in accord in their treatment of the mathematical side of elementary physical chemistry, but they are in substantial agreement as to what topics should be included and what should be omitted from an elementary course. The courses given by both departments include most of the standard topics to be found in the index of any recognized elementary text except that thermodynamics is omitted entirely. It is our feeling that the study of physical chemistry from the thermodynamic standpoint should be put off until the student has had a chance to find out what the subject matter of physical chemistry consists of. This topic is therefore postponed until the senior course mentioned under (*d*). Both departments agree also in the necessity for having the student work out numerous problems in connection with his elementary work. The chemistry department assigns about two hundred problems during the year. The biochemistry department assigns about 450 problems, some of which are non-mathematical.

The courses offered by the biochemistry and the chemistry departments for students in group *b* differ considerably in: (*1*) the order in which topics are taken up, (*2*) the illustrations cited for the various topics, (*3*) the reference books used, and (*4*) the amount of laboratory work required. These differences reflect partly the differences in personalities of the instructors (each feeling free to give his course in the way in which he feels he can do it best) and partly the previous training of the members of the class. They do not affect the general content of the two courses nor the basic aims which underlie them. The biochemistry department gives three lectures and one two-hour conference per week during the first semester on the general laws and theories of physical chemistry. This is followed in the second semester by two lectures and one three-hour laboratory period per week, covering the more specialized topics such as colloids, pH, buffers, potentiometry, surface tension, viscosity, electrophoresis, electroendosmose, membrane potential, electrometric titration, kinetics of enzyme reactions, photochemistry, etc. Practically all the illustrations used in the lectures come from the fields of biology, biochemistry, and agriculture, since these are fields of primary interest to the students. Most of these illustrations are but rarely found in the textbooks in physical and physiological chemistry in common use. During the first semester Chapin's "Second Year College Chemistry" is used as a text, supplemented by frequent references to Findlay's "Physical Chemistry for Students of Medicine," and Philips' "Physical Chemistry; Its Bearing on Biology and Medicine." During the second semester the text is Kruyt's "Colloids" supplemented by Pauli's "Proteins," Loeb's "Proteins," Clayton's "Emulsions," Cowdry's "General Cytology" and Hoeber's "Physikalische Chemie der Zelle und Gewebe" (translated by the instructor and made available as a reference to the students in typewritten form). Required laboratory work is confined to three hours per week during the second semester. Additional laboratory work may be elected.

The chemistry department gives three lectures per week during the first semester and two lectures per week in the second semester. A three-hour laboratory period per week is given throughout the year. The lectures of the first semester include atomic structure theory, the three states of matter (with considerable emphasis on the crystal structure of solids), colloids and surface phenomena, osmosis. Those of the second semester include chemical equilibria, thermochemistry, chemical kinetics, theory of solutions, electrolytic conduction, electrolytic equilibrium, electromotive force, pH, photochemistry. Since the lectures are also attended by students in metallurgy, ceramics, geology, chemistry, chemical engineering, and physics, an effort is made to draw in illustrations of physico-chemical principles from all these fields. This means that the pre-medical students do not have a chance to get so many illustrations from the biological sciences as do those in the course given by the biochemistry department. This is to a large extent, however, offset by the breadth of information which they gain by hearing in the same lecture illustrations from their own field and from two or three other fields. It is the experience of the chemistry department that the premedical men are as interested in the properties of metals as deduced from crystal structure studies as they are in learning that crystal structure studies indicate a fundamental difference in the type of union between the atoms of an organic molecule (such as their drugs) and the atoms of most inorganic compounds (such as NaCl). They seem as interested in monomolecular oil films on water or on the metals of a bearing as in the monomolecular layer of the stabilizing agent of an emulsion. As in the case of the course given by the biochemistry department, the illustrations used in the course given by the chemistry department come from many sources. They cannot be found in any one book, nor even in any ordinary collection of books. No regular text is used in connection with the lectures. The students are all required to use Getman's "Outlines of Theoretical Chemistry" because it contains so much material that they stand a good chance of finding in it the topic which they need to look up. No regular assignments are made in Getman, but whenever possible the statement is made in the lecture that the ground covered will be found in Chapter — of Getman. Premedical students are advised to use Bayliss' "General Physiology" as an additional reference. Knox's "Physico-Chemical Calculations" is required as a source of problems. The laboratory work, using Findlay's "Practical Physical Chemistry" as a text, consists of one three-hour period per week throughout the year. In addition to the text, mimeographed sheets are furnished for each experiment. These sheets are partly intended to fill the gap between the laboratory manual and the actual experiments which we assign, but they are also intended to give the student an idea of why he is asked to do that sort of experiment at all, and to show him the importance of that type of laboratory work in industry, medicine, or research.

Group (*c*).—This group includes juniors in metallurgy, ceramics, mining, geology, and electrochemistry. Although these men have all studied calculus they are, with few exceptions, distinctly non-mathematical in their general mental habits. All of these students come from other schools in the college and the amount of physical chemistry which they are required to take is prescribed by their own schools. For instance, metallurgists take all the lectures throughout the year but no laboratory work; ceramists take only the lectures of the first semester, so that they get no instruction in the theory of solutions, electro-chemistry, etc.; geologists and electrochemists are required to take both lectures and laboratory work during the first semester. These students all take their work along with the premedical men and with the juniors of group (*d*).

Group (*d*).—These men are all enrolled in the physics, chemistry, physical chemistry, or chemical engineering curricula. These students all have mathematics through calculus and most of them through differential equations. From the very nature of their work it is to be assumed that all of them have had considerable experience in using mathematics. During their junior year these men are all given the laboratory work and lectures already described for our pre-medical students under (*b*). This course serves to give them an idea of physical chemistry as a whole. It is the equivalent of the average undergraduate college course in the subject. We give as the aim of their junior course that, (*1*) the laboratory work is to give them a facility in making physico-chemical measurements without which they cannot hope to hold a job at all, and (*2*) the lectures and problems are to give them a knowledge of the theory without which they cannot hope to hold a worthwhile job.

The field of physical chemistry is already so large and is growing so rapidly that a student cannot learn in one year all that his prospective employer expects him to know. We have therefore scheduled a second year of work for our senior chemists, chemical engineers, physical chemists, and physicists. These men are already supposed to know from their work in the junior year what physical chemistry is about. They are supposed to have acquired a "feel" for the subject. They are now ready for a good stiff course which uses the junior course as a foundation, and which goes beyond the ordinary undergraduate work. During their senior year, therefore, they are required to cover the subject from the standpoint of thermodynamics. The course follows the general content and treatment of Noyes and Sherrill. The junior course has as its aim the student's immediate future; the senior course has as its aim the student's ultimate advancement.

The junior and senior courses not only give a good training for the student who must leave the university at the end of his senior year, but they also serve as a good foundation for his more fortunate brother who goes on to graduate work. The senior course goes beyond the undergraduate training in most universities. For this reason it carries graduate credit for the benefit of men coming to our Graduate School from other institutions. During the past year, one-third of the men taking our senior course were graduate students. They represented undergraduate work done in thirteen first-class colleges and universities. So far, not one of our graduate students from other institutions has had, as an undergraduate, the equivalent of the course described above. This may leave us open to the accusation of forcing our undergraduates too far. But we have to remember that each year, as our knowledge of physical chemistry grows, industry demands a better trained raw-recruit than the year before.

Such a course leads naturally to a general mathematical graduate course which covers in one year the equivalent of Taylor's two volumes, except that somewhat more attention is given to the mathematics of the Debye-Hückel theory of solutions of electrolytes. The course might well be called "The application of physics and mathematics to chemical problems." It is one of the aims of the course to help the students overcome the natural fear which any normal human being has of a page full of equations and to train them to read such a page easily with the aid of a pencil and a piece of paper. In this way we try to prepare them for a time, possibly ten or fifteen years hence, when by reason of changing conditions, the ability to read a mathematical article in physical chemistry will be a prerequisite to advancement in their chosen profession. For graduate students who present physical chemistry as their major subject the general mathematical course is followed by specialized courses in x-rays and crystal structure, colloids, catalysis, phase rule, atomic theory, spectroscopy, microchemistry, etc. For graduate students in bio-physical chemistry the department of biochemistry offers additional courses in physical chemistry of the cell and in bio-physical measurements. These courses not only lead to advanced degrees in bio-physical chemistry when supplemented by physical chemistry courses offered by the chemistry department and by additional work in mathematics, but they also serve to give physicochemical training to graduate students in agriculture.

We do not wish to take the ground that the solution given above to the problem of teaching physical chemistry is the only possible solution, or even that it is the best possible solution. We do feel that it is a workable solution which apparently fits our peculiar conditions. We have outlined our solution rather completely in the hope that it may be of value to other institutions which have equally complex problems. It is inevitable that the men we graduate this year will some day have to compete with younger and still more highly trained men. This implies that any proper solution of the problem of teaching physical chemistry must take into account the obligation to give our students such a training in mathematics and science that this year's class can fill for itself, by home study, the gap between physical and bio-physical chemistry as we know it today, and the physical and bio-physical chemistry that will be fifteen years from now.

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NUTRITIONAL LEG-WEAKNESS IN POULTRY

Factors Influencing the Rate of Ossification in Growing Chicks[1]

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Previous studies[1] in this laboratory, using rats as the experimental animals, have led us to believe that the bone-ash method is very useful in determining the efficiency of bone ossification. Furthermore, since x-ray and line-test data did not lend themselves readily to quantitative interpretation, it was thought that the determination of bone-ash might be useful in arriving at quantitative conclusions, provided sufficient data could be collected, using normal and rachitic animals at various ages. Unpublished data obtained during the past two years support this assumption, since in many cases we have obtained definite proof of increased ossification as evidenced by the line-test when in reality the bone-ash determination showed little or no bone deposition. On the other hand, bones from different animals have been compared where the line-test showed no marked differences in ossification, while the percentage of bone-ash in rat femora differed as much as seven per cent.

The experiments described in this paper were designed to make a similar study, using baby chicks, and determine the rate of bone deposition during the first eight weeks of life under various environmental conditions.

Two sets of experiments are described in this paper. The first experiment was started in March, 1927, and continued for

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eight weeks with the view of obtaining information relative to the rate of ossification as influenced (1) by a leg-weakness producing (rachitogenic) ration, (2) by sunlight transmitted by a window glass substitute, (3) by direct irradiation with a quartz mercury vapor lamp, (4) by Newfoundland cod liver oil. The second experiment was conducted later in the year, starting on May 31 and terminating on July 25. During this period studies were made with the view of determining the rate of bone development as influenced by (1) a normal poultry ration, (2) by sunlight transmitted through a window glass substitute, (3) direct irradiation by the carbon arc lamp, and (4) by a Norwegian cod liver oil.

EXPERIMENTAL

Four hundred day-old White Leghorn chicks were used in each experiment, each experimental group containing one hundred chicks at the beginning of the investigation. Each group was placed in a specially designed brooder pen equipped with an electric brooder and the mash troughs were equipped with grids to prevent the scattering of feed.

The windows of the poultry house were coated with Bon Ami paste to diffuse the light transmitted by the window glass and certain windows were covered with burlap to reduce the amount of light. The experimental groups which were subjected to transmitted sunlight were placed in a specially constructed sun parlor equipped with the window glass substitute to be studied. This sun parlor was attached to the southeast portion of the poultry house. The groups which were irradiated directly with the ultra-violet lamps were so placed that the light rays could not affect the other pens.

The basal (rachitogenic) ration was that described by Hart and co-workers[2] and consisted of ninety-seven parts of yellow corn meal, two parts of calcium carbonate and one part of sodium chloride. The Wisconsin workers and others who have used this ration have fed skim milk ad libitum. We followed the suggestion of Kennard[3] and mixed ten pounds of powdered skim milk to one hundred pounds of the basal ration mentioned above. Sufficient data have been obtained in our own and in other laboratories to show that the ratio of calcium to phosphorus, in the feed, is of great importance in bone formation. We felt that the inclusion of the powdered milk in the ration should eliminate fluctuations in the calcium-phosphorus ratio, which might not be true if individual birds were allowed to eat varying amounts of skim milk without reference to the amount of basal ration ingested. To eliminate the possibility of spoilage and subsequent vitamin oxidation in storage, the experimental rations were prepared each week. All birds were leg-banded and weekly growth and feed-intake records were collected throughout the experiments.

Two sources of ultra-violet light were available in the experimental poultry house—a quartz mercury vapor lamp (known as the Uviarc Poultry Treater) and the other a blue flame carbon arc lamp. The former was operated at a distance of thirty inches (from lamp to floor of pen) and the latter was operated at a distance of forty inches.

Line-tests and bone-ash determinations were made on the tibiae at the end of each week. One tibia from each chick was reserved for the line-test, which consisted of splitting the bone and placing the parts in a ten per cent. solution of formalin. These were washed with distilled water and transferred to a one and one-half per cent. solution of silver nitrate. After subjecting the bones to this solution for one minute, they were washed, exposed to light (to darken) and examined under the low power binocular microscope (using a camera lucida attachment, when drawings were to be made).

All ash determinations were made on the remaining tibiae. Owing to the natural fluctuation in fatty materials in bone, it has been found advisable to extract all bones with alcohol and ether. This is done by drying the bones at 95-100° C. for twenty hours, which makes them porous and permeable to alcohol and ether. It was found that in alcohol extraction (using 95 per cent. alcohol), in a Soxhlet extractor for fourteen hours, followed by extraction with ether for a similar period, was sufficient to remove the lipins completely. The mineral content of the alcohol-ether residue is not of importance in this work, since we are attempting to account for that portion of the inorganic material which has actually formed bony tissue. After extraction the bones were dried to constant weight, incinerated in an electric muffle furnace and the amount of ash was calculated to per cent. on the basis of lipin-free and water-free bone. Although individual analyses were made on each chick, the results reported represented the average of six analyses.

The First Experiment, March 15 to May 9, 1927

Four hundred day-old baby chicks were placed on experiment March 15, 1927. They were divided into the following experimental groups, each consisting of one hundred chicks:

Group 1. Basal (rachitogenic or leg-weakness producing) ration.

Group 2. Cel-o-glass group. Basal ration supplemented with sunlight transmitted by Cel-o-glass.

Group 3. Irradiated group. Basal ration supplemented by ultra-violet light from the Uviarc Poultry Treater for fifteen minutes daily, at a distance of thirty inches. Birds and feed were irradiated simultaneously.

Group 4. Cod Liver Oil Group. Basal ration supplemented at the rate of two pounds of Newfoundland cod liver oil per one hundred pounds of ration.

At the end of each week six chicks, picked at random from each pen, were killed by decapitation and the bones were removed and treated as described above.

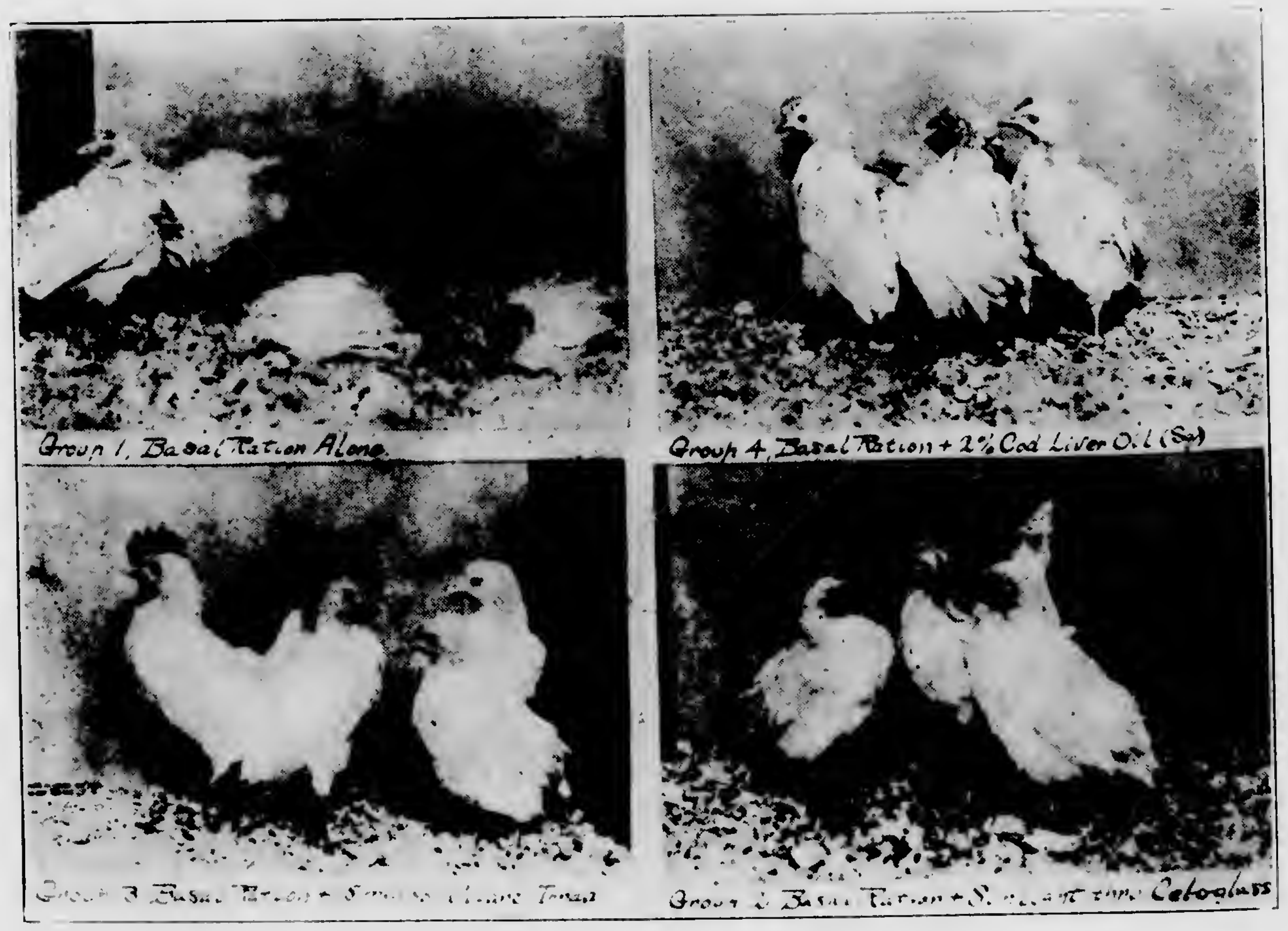

Group 1, Basal Ration Alone
Group 4, Basal Ration + 2% Cod Liver Oil (Sq)
Group 3 Basal Ration
Group 2 Basal Ration + Sunlight thru Celoglass

FIGURE I.

Typical birds of each group in the first experiment. Photographed at six weeks of age.

The Second Experiment, May 31 to July 25, 1927

The same number of birds were used in this experiment as in the one just described. Four groups of one hundred chicks each were divided according to the following treatments:

Group 1. Typical Poultry Ration. This ration is known as the Pennsylvania State College (1927) All-Mash Ration and, as made up in the poultry plant, consisted of 400 pounds of corn meal, 165 pounds of bran, 150 pounds of middlings, 100 pounds of dried buttermilk, 50 pounds of fish meal, 20 pounds of bone meal, 100 pounds of alfalfa leaf meal, 5 pounds of limestone, 5 pounds of cod liver oil and 5 pounds of salt.

Group 2 Vitaglass Group. Basal (leg-weakness-producing) ration supplemented with sunlight transmitted through Vitaglass.

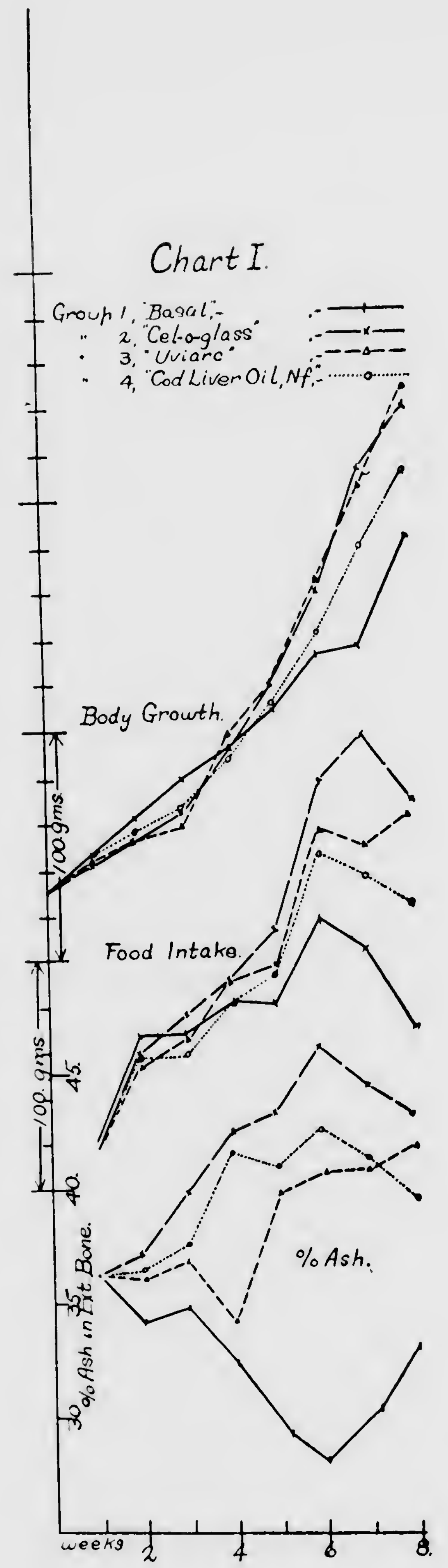
Group 3. Irradiated Group. Basal ration supplemented for 15 minutes daily by ultra-violet light from a blue-flame carbon arc lamp (with iron-cored electrodes) at a distance of forty inches from the burner. Birds and feed were irradiated simultaneously.

Group 4. Cod Liver Oil Group. Basal ration supplemented at the rate of two pounds of Norwegian cod liver oil per one hundred pounds of ration.

Discussion of the First Experiment

In order to conserve space and arrange the data so that the results obtained may be more easily visualized, they have been arranged in the form of charts rather than in tabular form. Chart I summarizes the growth, food intake and bone-ash records for the first experiment, while Chart II summarizes the data obtained in the second investigation.

While the charts are self-explanatory, it may be pointed out that the growth curves show that Group 1 (Basal), Chart I, did not grow as well from the fourth to the eighth week as the other groups. Furthermore, Group 2 (Cel-o-glass) and Group 3



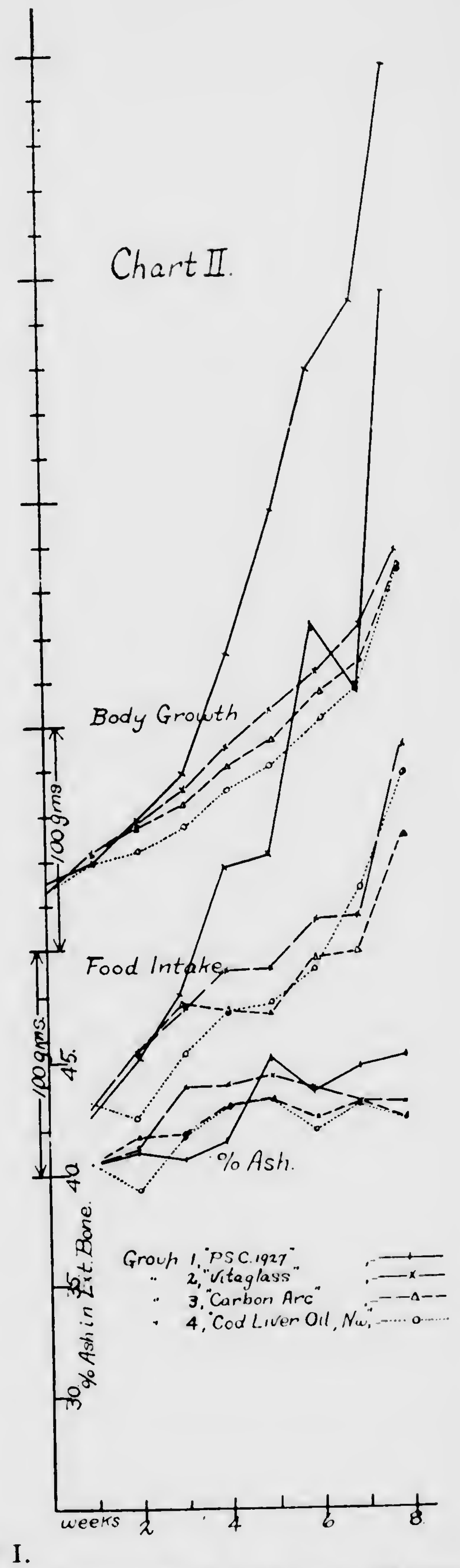


CHART I.

Showing variations in body weight, food intake and bone ash (March 15 to May 9, 1927).

CHART II.

Showing variations in body weight, food intake and bone ash (May 31 to July 25, 1927).

(Uviarc) grew at the same rate, which was somewhat superior to that of Group 4, which received cod liver oil. The curves indicating the average food intake per bird per week do not differ materially until the middle of the experimental period, when the food intake curves arrange themselves in about the same order as those representing growth, i. e., that the light-treated groups ate somewhat more food than the one receiving cod liver oil and the basal group consumed less food per bird per week than any of the other groups on experiment.

Leg-weakness (Fig. 1) was confined to Group 1, which received the Leg-weakness Producing Ration. The symptoms were most pronounced during the fifth week of the experiment, and by the sixth week ninety-five per cent. of the birds were afflicted with the disease.

Examination of the curves representing the weekly averages of the bone-ash determinations, shows that all of the birds started the experiment with about 36.3 per cent. of bone ash in their tibiae. Before the experiment had progressed far, however, differences could be noted in the efficiency of ossification as represented by the average of the bone-ash determinations.

Line-tests appeared to be most significant at or about the fifth week of the experimental period, so far as the bones of the chicks in Group 1 were concerned. In the other groups the diaphyses appeared to be uniform in structure and the condition of the osteoid tissue was regular and dense.

The bone-ash curves show that definite differences exist so far as quantity of mineral matter, deposited per bird per week, is concerned. Group 2 (Cel-o-glass) showed superior bone depositing ability from the start of the experiment, reaching a maximum of 46.2 per cent. of bone-ash at the sixth week. Cod liver oil (Group 4) ranked next to Cel-o-glass in efficiency and irradiation by the Uviarc lamp was somewhat less efficient than the cod liver oil during the early stages of the experiment. By the seventh week, however, Groups 3 and 4 were practically identical, so far as the ash content of the tibiae was concerned.

DISCUSSION OF THE SECOND EXPERIMENT

The growth curves in Chart II reveal little of importance other than that Group 1, receiving the College All-Mash Ration, grew at a rapid rate as compared with the other groups. This, however, is to be expected, since the Basal Ration is composed almost entirely of corn and in no sense can be considered a good growing ration. It should be noted, also, that the chicks in the second experiment averaged somewhat less in body weight at the time of hatching than those used in the previous investigation.

Comparison of the groups receiving the supplemented Basal Ration shows nothing of importance. All groups grew at about the same rate, regardless of the nature of the supplementary treatment. If any superiority can be detected, so far as growth is concerned, it would appear to be in favor of the light treated groups, the cod liver oil being the least efficient. However, by the end of the eight week period no differences can be noted. What has been said of the growth responses, in the second experiment, also applies when food intake is considered, the two sets of curves arranging themselves in about the same order.

When we study the bone-ash curves we find a similar situation. In general, the tendency to deposit calcium and phosphorous was greatest in the group which received the best ration, from the standpoint of chemical composition, i. e., the Penn State All-Mash Ration. No outstanding differences can be noted when the vitaglass, carbon arc and cod liver oil treatments are compared. No leg-weakness existed in any group in this experiment.

GENERAL DISCUSSION

The reader's attention should be called to the fact that, in all of the experiments where light was used as the supplement, no attempt was made to differentiate between the physiological effect of light on the birds and the activation of the feed by ultra-violet light. Our object in this was to approximate practical feeding conditions as closely as possible so far as artificial light and transmitted sunlight are concerned.

Chart III summarizes the hours of sunshine per week throughout each experiment. The birds in the last experiment (May to July) received 247.5 hours of sunshine as compared with 193.6 hours for the first experiment (March to May). In spite of the fact that the birds in the later experiments received an excess of 53.9 hours of sunshine, no superiority in bone deposition can be noted. According to the Bureau of Standards[12] new vitaglass transmits about 50 per cent of the ultra-violet light while new Cel-o-glass transmits about 20 per cent.

It will be noted that the birds hatched early in the season (March) weighed more (29 to 30 grams) at 48 hours of age than those hatched in May. The body weights of the latter ranged from 26 to 29 grams. Buckner[1], Hart and co-workers[5], Wood[6] and Russell and Massengale[7] have shown that the average weight of baby chicks of the same breed and age is about 35-45 grams.

The importance of strain, age of parent stock, seasonal influences, such as temperature and light intensity, storage of eggs used for hatching, etc., are of the utmost importance and should be studied. It remains for the poultry investigator to try to standardize his experimental birds just as the rat breeders have striven to produce uniform and standardized rats.

Goodale[8] states that seasonal effects on growth can be noted by studying subsequent hatches throughout the season. He contends that growth response seems to be greatest in the early hatches and seems to diminish as the hatching season advances.

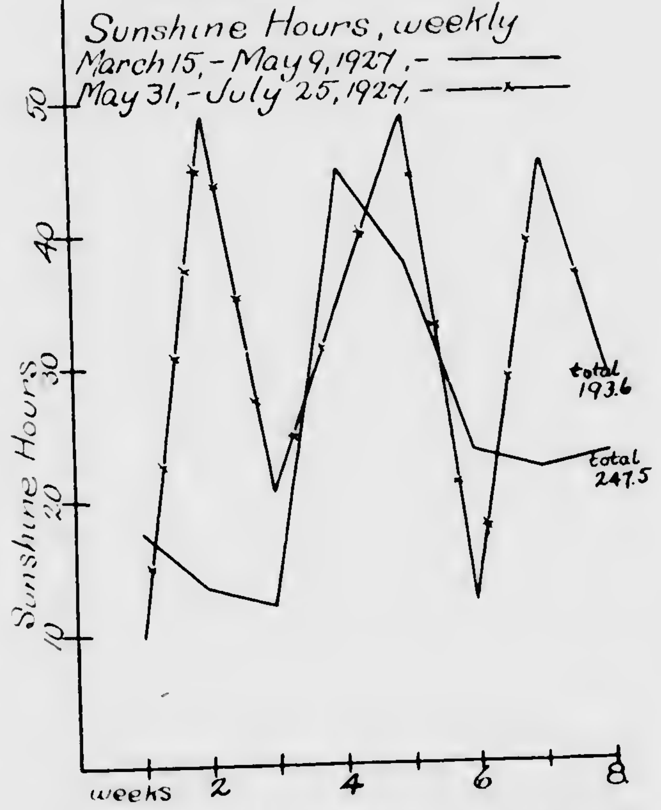


Chart of weekly sunshine hours as obtained from Weather Bureau Reports at State College, Pa.

The greater amount of bone-ash in the bones of the 48-hour chicks in May, as compared with those hatched in March, would lead us to infer that the parents were able to transmit a greater amount of the anti-rachitic factor to the egg in May than would have been possible in March, due in all probability to better light conditions. This inference receives support in the work of Hart and co-workers[9, 13], and of Hughes and co-workers who found that the irradiation of laying hens had a definite effect on the hatchability of the eggs.

McCollum and co-workers[10], Cavins[11] and Dutcher and co-workers[1] have shown that fasting often brings about an increase in inorganic blood phosphorus when the animal has been subsisting on a rachitogenic ration. This view is further substantiated by the deportment of the chicks in Group 1, Chart I. Appetites were good and food intakes increased until the sixth week of the experiment and during the following two weeks the food intake per bird per week decreased very appreciably. It was at this point that the symptoms of leg-weakness were most marked. It is most interesting to note nature's attempt to prevent the further development of the disease by bringing about the loss of appetite at the point where the disease is most serious. At this point the tibiae increased from 28 to 33 per cent of bone-ash in two weeks.

SUMMARY

Experiments are described in which the bone-ash of White Leghorn chicks has been determined for the first eight weeks of life under various conditions of feeding and light treatment with the view of collecting data which may be used in future studies relative to the quantitative determination of the ossifying potency of anti-rachitic substances and the following observations may be made.

1. That the fluctuations in bone-ash in the tibiae of growing chicks are quite similar to those we have obtained in similar studies on the rat.

2. That the data obtained should enable us to repeat this type of experiment, using graduated amounts of rachitic foods and graduated amounts of light until we find that amount of food or light which will prevent the development of leg-weakness and maintain the bone-ash (for five to six weeks) at the level possessed by the newly hatched experimental birds at the beginning of the experiment.

3. The proposed application of the data obtained in this study should enable us to make quantitative comparisons of poultry feeds and various sources of ultra-violet light, as well as to study those environmental conditions which affect the transmission of calcifying factors to the egg.

4. While the experiments described in this paper were not planned with the view of obtaining detailed quantitative information at this time, the results indicate that irradiation (for 15 minutes daily) with the quartz mercury vapor lamp and with the blue-flame carbon arc lamp and by means of sunlight transmitted through Vitaglass and Cel-o-glass produced results in growth response and skeletal development comparable with those obtained with Newfoundland and Norwegian cod liver oils.

Work is now in progress with the view of using the data obtained in this report to make a quantitative study of the bone depositing potency of some of our common poultry feeds.

ACKNOWLEDGEMENTS

The writers acknowledge their indebtedness to Mr. Paul S. Margolf, Poultry Superintendent, for his interest and coöperation in this investigation; to the Cooper-Hewitt Electric Company, Hoboken, New Jersey, for the use of the Uviarc Poultry Treater; to the National Carbon Company and Dr. M. J. Dorcas for the use of the blue-flame carbon arc lamp; and to the Vitaglass Corporation, New York City, for the Vitaglass used in the second series of experiments.

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Reprinted from SCIENCE, March 8, 1929, Vol. LXIX, No. 1784, page 276.

VITAMIN B TERMINOLOGY

IN SCIENCE of August 31, 1928 (Vol. 68, No. 1757, pages 206–209) was published a summary of the various proposed systems of vitamin B terminology then under consideration by the committee on vitamin B nomenclature of the American Society of Biological Chemists.

The various proposed systems of terminology eventually narrowed down to three, which seemed in the minds of the committee to be most likely to satisfy the majority of workers in the field of vitamin research.

Summarized briefly, the three systems of terminology were as follows:

(1) *The British suggestions.* The British investigators recommended, as a temporary expedient, that the letter ‘‘B’’ be used to designate the complex, ‘‘B_1’’ to identify the heat-labile factor, and ‘‘B_2’’ to refer to the heat-stable factor or factors.

(2) *The Sherman suggestions.* Dr. Sherman suggested that the term ‘‘B’’ be used to refer to the complex with the hope that it would eventually receive ‘‘honorable retirement’’ and that the letters ‘‘F’’ and ‘‘G’’ be used in a permanent system of nomenclature, in place of ‘‘B_1’’ and ‘‘B_2,’’ respectively, which had been adopted as temporary terms by British workers.

(3) *The McCollum suggestions.* Dr. McCollum, for reasons enumerated in the former committee report, suggested that the term ‘‘B’’ be retained but restricted to refer to the heat-labile factor, and that Sherman's ‘‘G’’ be used to designate the more heat-stable factor.

After considerable correspondence the committee have voted to make the following recommendations:

(1) That the term ‘‘Bios,’’ as suggested by the British workers, be retained to denote the factor or factors encouraging the rapid growth of yeast cells.

(2) That the term ‘‘B’’ be restricted to designate the more heat-labile (antineuritic) factor.

(3) That the term ‘‘G’’ be used to denote the more heat-stable, water-soluble, dietary factor, called the P–P (pellagra-preventive) factor by Goldberger and associates, and which also has to do with maintenance and growth.

(4) That the naming of newly discovered dietary factors, by other than descriptive terms, should be discouraged until their identity is established beyond question.

(5) That a committee on vitamin nomenclature be appointed by the American Society of Biological Chemists and that this committee, in cooperation with the British committee and similar committees on the continent, endeavor to act as a clearing-house for questions in vitamin terminology, and that this committee be empowered to name new factors when their identity is established.

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Vol. XXVII, No. 4, April, 1929

STUDIES OF CERTAIN PHASES OF THE INTERRELATIONSHIP BETWEEN SOIL AND PLANT: I. AVAILABILITY OF MINERAL PLANT NUTRIENTS IN RELATION TO THE DEGREE OF DISPERSION[1]

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Received for publication December 29, 1928

A. INTRODUCTORY

The subject matter of this paper deals with a field of research that, although of the greatest importance, is still in a state of flux. Notwithstanding the existence of a voluminous literature on the permeability to and absorption of substances by living animal and plant cells, there is little exact knowledge. And to the complex of factors now to be considered that of colloidal behavior must be added.

Colloidal solutions have been *arbitrarily* defined on the basis of the size of the dispersed particles ($1\mu\mu$ to 100–$200\mu\mu$). There is, however, no infallible test for colloidality. Even the ultra microscope and cataphoresis tests may fail under some conditions and with some colloids (82). Dialysis experiments, moreover, may not always be specific, for artificial semi-permeable (collodion) membranes have been prepared that are permeable to one crystalloid (e.g., NaCl), but not to another (e.g., $NaNO_3$) (7, 8); to starch and dextrin, but not to night blue and other colloidal dyes; to colloids but not to crystalloids (45, 46).

1. *Theories of permeability*

It is not definitely known what makes a membrane semi-permeable. According to Kahlenberg (45) an absolutely semi-permeable membrane exists only in theory. From their comprehensive studies on the conditions governing the permeability of certain physiological membranes for various *electrolytes*, Michaelis and Weech (59) conclude that it is fallacious to speak of a *definite permeability* of any type of membrane for electrolytes, since in their experiments the behavior towards the passage of electrolytes was found to depend on a variety of conditions. Thus, these investigators showed that by changing the character and magnitude of the force driving the ions across the membranes and, according to the history of previous treatment of the membrane, the whole character of the so-called specific permeability for ions of the membrane could be varied *without any substantial change in the structure, chemical composition or pore size of the membrane.* And Höber (39) is of the opinion that membranes possess both a "physiological" as well as a "physical" permeability, because the mechanism of penetration into membranes by the different classes of substances, such as organic substances, salts, acids, bases and water, appear to be so distinctly different with each group. The older definitions, therefore, of semi-permeable membranes break down entirely.

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[1] Publication authorized by the Director of the Pennsylvania Agricultural Experiment Station as scientific paper No. 462.

All theories of the permeability of *living* cells assume the existence of some sort of a "membrane," although differences of opinion exist as to its nature. The more prominent of the older conceptions of the factors determining the penetration of substances into living cells are Overton's "lipoid" theory (68) and Clowes' conception of the cell membrane as an "emulsion" (13); but it has been urged (41) that no one theory is capable of explaining all the peculiarities of cell permeability such as, for example, the penetration of water-soluble substances like sugar and which are not lipoid-soluble. Moreover, most of the theories break down in accounting for functional changes in permeability, that is, the permeability of the plant cell is not constant. Nevertheless, most authorities agree that there can be little doubt that "lipoid" solubility is a property favoring ready entrance into living cells, the sole exception to the rule known at present being the cases presented by a *colloidal dye* in the presence of plant cells (39).

2. *What constitutes true colloidal behavior?*

One of the main difficulties to be faced in an evaluation of the factors determining whether or not colloids can be absorbed by plants is in characterizing what constitutes true colloidal behavior. Although colloids have been arbitrarily defined on the basis of the size of the dispersed particles, there is no agreement on what constitutes true colloidal behavior. Loeb (55) premises that ampholytes like the proteins show colloidal behavior only when the protein ions are prevented from diffusing through those membranes or gels that are readily permeable to the ions of ordinary salts and that the distinction between crystalloids and colloids, as Graham (29) conceived the difference, is not tenable. If all ampholytes behave like proteins (as doubtless they do), then, according to Loeb's view, they are true colloids only at the isoelectric point, i.e., when they exist as non-iogenic substances. The ions of Loeb, then, that show true colloidal behavior are the same as the micellae of McBain that originate through the electrolytic dissociation of neutral colloidal systems.

If, as it would appear, colloidal behavior is a surface phenomenon, the clearest conception of the behavior of colloids is obtained, as Wilson suggests (96), by regarding the particles as having more of their valences directed from the molecule and, therefore, unsatisfied (i.e., residual valency). The consequence of the existence of such free valences will be to cause substances approaching their surfaces to combine and give up their charge to the colloid. The colloid may also acquire its charge by ionizing itself (22).

As Oakley (65) well expresses it, the colloidal condition may well be conceived as a compromise due to the differences in the affinity of the un-ionized and ionized groups for water, the boundary of the ionized ions being conditioned by the balance existing between the osmotic and electrometric forces. The membrane defined by the limits of this boundary will be governed by the Gibbs-Donnan equilibrium, the non-diffusible ions being the anions or cations that are partners to the colloidal cations or anions, resulting in a potential difference (P.D.) between the surface layers and the bulk of solution. This P.D. is found by equating the electrical work done by moving one equivalent of ions across the membrane to the work done against osmotic pressure, i.e., to $\frac{R.\,T.}{F}\log y/x$, where y/x are the concentrations of the negative ions on the two sides of the membrane.

3. *On dialysis experiments*

But all of these, conceptions, of colloidal behavior fail to explain much that is pertinent to our problem. For example, does dialysis increase or decrease the stability of sols? Hardy (35) maintains that dialysis as a means of isolating unstable or insoluble hydrous colloidal matter is open to criticism on the grounds that it progressively removes adsorbed stabilizing (peptizing) ions, thus destroying equilibrium conditions. The experiments of Thomas and Frieden (83) furnish an excellent example of this. There is a wide gap in our knowledge as to the causes operative in producing stability of sols and, therefore, of their exact composition. Take ferric hydrosol as an example. Thomas and Frieden (83) claim that the ferric oxide sol is represented by the formula xFe_2O_3, $yFeCl_3$, and that it exists in solution as the complex ions $xF_2O_3 \cdot Fe^+$ and Cl^-. But additional corroborative evidence is lacking to support this view. Indeed, Sorum's investigations (78) established beyond much doubt that stable hydrosols giving no test for Cl^- can exist. From Beans and Eastlack's work (3) it might be deduced that the ferric oxide sol in Sorum's experiments is stablized by the small amount of Fe in solution (for the oxide is soluble to the extent of 1 p.p.m.), or, possibly, it might be stabilized by the hydrogen ion from the ionization of the water on its surface.

From the foregoing it will be realized how fragmentary is our knowledge of the facts of permeability and how impossible it is in the present state of our knowledge to formulate any immutable laws of membrane action. This condition, however, may not prevent us from critically examining in detail the available experimental evidence bearing on our problem, to weigh the facts and to interpret them according to the scientific method. This we shall proceed to do.

B. EXPERIMENTS WITH ARTIFICIAL MEMBRANES, ISOLATED CELLS OR TISSUES AND ABNORMAL PLANTS

1. *Artificial membranes*

a. Gurchot's theory of colloidal penetration. The suggestive researches of Gurchot (31), indicating the mechanism of the reversible permeability of copper ferrocyanide membranes, have given additional information on the possible mechanism of penetrability. His copper ferrocyanide membranes give all evidences of behaving as a dynamic system. He concludes that the whole question of the action of his membranes involves adsorption, coagulation and peptization. That some form of adsorption, or, perhaps, solution of the septum takes place is now widely accepted. This is the basis of Overton's theory (67) and is very strikingly demonstrated in Kahlenberg's experiments (45, 46) with rubber damask and lanoline silk membranes and in Overton's own experiments (67) on animal and plant cells.

It is possible that a satisfactory working hypothesis of the behavior of *artificial* membranes may be worked out in the future on the basis of reduction or neutralization of the charge upon their colloidal surfaces. Penetration of the membrane by colloids would, if such a theory is substantiated, be due to membrane coagulation due to selective adsorption as a result of differences in potential set up at the interface between the solid and liquid phases in accordance with the Gibbs-Donnan law. It would serve to explain why substances pass through a membrane at certain times that do not do so normally. Gurchot's theory of membrane action does not imply that penetration is always accomplished by means of selective adsorption and coagulation. On the contrary, as he points out, under some conditions a membrane may act like a sieve for salts in dilute solution, because in this case they will, if the solutions are dilute enough, go through the membrane without coagulating it. This is entrance by osmosis. But when the concentration is raised the charge of the membrane will, if the cation is absorbed more than the anion, be neutralized and coagulation will, therefore, follow, in which case the salts or ions pass through by diffusion as through a dialyzing membrane. Indeed, Weech and Michaelis (92) are disposed to account for the difference in the diffusion rates of the non-electrolytes examined by them to the varying sizes of the pore channels of the membranes.

From Gurchot's results it can scarcely be questioned that artificial membranes, like the potassium ferrocyanide septums used in his experiments, may, under certain conditions, permit the diffusion of colloids; but the experimental demonstration and proof of colloid penetration in the case of normal living cells, as will be shown, is less convincing.

b. Equilibrium of colloidal and molecularly dispersed phases. There is, moreover, another possible mechanism that must not be overlooked. If, as it is reasonable to suppose from the hydration theory (96), that a fractional portion of all sols are in true molecular solution, penetration of colloids into cells would take place, preferably by the entrance of that portion of the substance which is in true solution. The disturbance of the equilibrium produced by the diffusion of solute would enable more of the colloid to go into true solution. Evidence for this view is supported by the work of Foerdi and also of Fredenthal as recorded by Bechhold (4). From the observation that after ultrafiltering pure starch "solutions" a given fraction of the "solution" always passed through his membranes, the former concluded that for every concentration of the starch solution a balance existed between the larger particles and the molecularly dissolved starches. This view is upheld by Fredenthal who found that soluble starch produced a definite lowering of the freezing point (F.P.). We shall have occasion to discuss this conception later. But the experiments cited cannot be regarded as final, for marked differences existing in the membranes used by different investigators might vitiate comparisons unless the results are expressed in terms of "diffusion coefficients" (92). There is little doubt that differences in structure and composition of membranes, together with factors already stated (59) have contributed not a little to the difficulties presented in interpreting much of the experimental work on the ions present in the soil solution and which have been based upon dialysis experiments with collodion membranes. The confusing results on aluminum as a factor in soil acidity may be cited as an example (21, 43, 57).

2. *Living isolated cells and abnormal plants*

a. The absorption of fats. The work of Czapek (20) and Schmidt (77) on the penetration of fine emulsified fats is frequently cited in support of the theory of *colloidal absorption* by living cells. A critical investigation to test this theory was recently undertaken by Rhine (74). Schmidt's technique was repeated. When filter paper saturated with various oils colored with Soudan III were inserted into longitudinal incisions in starved pea seedlings, Rhine found that both *free fatty acids and neutral oils* entered the cytoplasm from the intercellular spaces and appeared in the cytoplasm in the form of small droplets. From these experiments he concluded that the penetration was a physical matter depending in part upon the viscosity of the oil. When, however, the tests were made with *emulsions of linseed fatty acid in water*, no penetration into the cytoplasm took place. Further investigations to explain this discrepancy disclosed the fact that the penetration of the fats and oils into the cells was due to a water deficit in the walls of the starved etiolated pea seedlings, for when tests were conducted with normal seedlings well supplied with moisture in air and soil no penetration took place.

Moreover, comparing the respiratory quotients in the hypocotyls of fatty and starchy seeds for the purpose of determining the type of food used by the germinating pea seedling, Rhine rightly concluded that the form could not be fat, since the fatty seed hypocotyls were being furnished carbon in the same state of reduction as were those of the starchy seeds. Other experiments showed that no diffusion concentration gradient of fats existed, nor were fats found "en route" in the cell walls in tissues through which formerly they were supposed to pass.

These critical investigations of Rhine appear to offer evidence that fat emulsions do not enter normal plant cells, but rigid experimental proof using animal cells has not been forthcoming.

Bechhold (4) is authority for the statement that fats are found in the truly soluble form (e.g., soaps) only at the time when they pass through the wall of the intestine, after which they immediately regain their colloidal condition. Unfortunately, he cites no experimental evidence. If his view is substantiated, the mechanism of penetration in such cases might be comparable with Witzemann's conception (97) of the catalytic function of the leucoplasts in the condensation hydrolysis equilibrium involved in the formation and mobilization of starch and glycogen, the equilibrium being controlled by the second law of thermo-dynamics.

b. The penetration of dyes. Such an authority as Jacobs (41) states that many of the investigations on the penetration of acid and basic dyes (colloidal as well as non-colloidal) are worthless, partly on account of faulty technique and partly due to too broad generalizations from results on specific types of cells. Of the investigations relative to the conditions determining the penetrability of dyes, Ruhland's work (76) acquired prominence because his conclusions challenged Overton's view that "lipoid solubility" was the greatest factor determining ease of entrance into living cells. Ruhland demonstrated beyond doubt that not only the basic or acid nature of the dye but also, in the case of plant cells at least, the size of the molecule was the important factor. Colloidal dyes did not penetrate whereas non-colloidal dyes passed through. Höber (38) and more recently Gaedertz and Wittgenstein (23) have also confirmed Ruhland's conclusions (76).

However, it would be dangerous to infer that, because certain dyes having large molecules do not penetrate living cells, this is the determining factor in penetration. Beyond a certain limit this does not seem to hold true. This is exemplified by the results of permeability experiments with certain organic substances in which it has been shown that the size of the molecule may be of less importance than the presence of non-polar groups (51), for it is known that the introduction of such non-polar groups as $-CH_3$, $-C_2H_5$, and $-C_6H_5$ in place of hydrogen into the fatty acids increases their permeability (41).

C. CULTURE AND FIELD EXPERIMENTS WITH PLANTS

1. Distinction between permeability and absorption

There is, in the literature, some confusion of the terms permeability and absorption. As Osterhout (66) has shown, the terms are not synonymous, because if ions are removed by reactions occurring in the cell or in the intercellular spaces, considerable absorption of the nutrient may take place, although the true permeability of the substance might be quite low. Accordingly, with the same permeability, the amount of substances absorbed may vary greatly, depending on conditions. In the following discussion on the absorption of nutrients relative to the degree of dispersion this distinction should be borne in mind.

2. The arguments advanced against the solution theory of plant nutrition

Comber's stimulating paper (14) on the possible rôle of colloids in plant nutrition has attracted considerable attention. This authority rightly objects to "the tendency among many soil chemists to take so many things for granted without inquiring scientifically and experimentally into their truths" (17). He feels convinced that the assumption that material must be in solution in the soil before it can be of any use to the plant is quite unproved. In modification of present views, Comber postulates that the colloids of the soil play an important rôle by being directly absorbed by plants through the formation of a one phase system between the colloidal coating of the plant root hairs and the colloidal coating of the soil particles. Hence, according to this hypothesis, part of the nutrients absorbed on the soil surfaces are mechanically incorporated by the root hairs. Nutrients may in this way be absorbed without the necessity of going into solution. If this view is true it would follow that diffusion of salts in the soil, especially those of phosphorus and of iron, would not be an important factor in plant nutrition. It should be added that an alternative hypothesis to that of the direct absorption of colloids is also put forward by Comber (14), viz., that the organic excretions of the roots may exert a dissolution effect on the particles.

Clearly, this conception has an important bearing on soil solution studies and soil analyses, on which account rigid proof should be forthcoming before it is accepted. The arguments advanced in favor of the proposed modification will now be critically examined.

a. The relation of the composition of the soil solution to the mineral elements taken up by plants. It has been maintained on the basis of observations of Hall (33) and of Ramann (73) that, since the ratio of certain mineral elements assimilated by plants to the water transpired is greater than the ratio of these same elements to the water in which they are dissolved in the soil solution, therefore, the crop does not derive all its mineral matter by the simple flow of the soil water by osmosis into the roots. Accordingly, the examination of the soil solution to ascertain what soluble substances are present is no criterion of what a particular plant can get out of the soil.

This conclusion cannot be valid, for, as will be shown, the premise is unsound: (*a*) Based on a calculation of the amount of K_2O taken up by a clover crop to the water transpired, Hall claims that the concentration of K_2O thus obtained, viz., 0.006 per cent, is greater than in solutions obtained from the soil. That such a limit may not hold generally is shown by many analyses of the writer (84) of the soil solution obtained by displacement methods (52,69) from a large number of orchard soils in Pennsylvania in which the K_2O content was found to be 0.0078 to 0.0084 per cent. (*b*) Additional evidence is furnished from the carefully controlled and exhaustive experiments of Muenscher (61) and also of Prat (72) which provide rigid proof of the fact that transpiration does not play an important rôle in the supply of nutrients to the plants. They have shown conclusively that the quantity of an element absorbed by a plant bears no relation to the water absorbed and lost by transpiration. In other words, Muenscher and Prat have proved that plants can absorb water and solutes differentially, the quantity of an element absorbed being dependent upon growth as influenced by humidity and light intensity, rate of transpiration and concentration of solution. Hoagland (37), moreover, has shown that the rate of absorption is also dependent on the nature of the ions.

These conclusions have also been experimentally substantiated by Curtis' investigations (18). Transpiration, therefore, cannot have much direct influence on the movement of solutes. Chemical change in the cells is of far greater influence than the mere mechanical effects of transpiration (19).

b. The absorption of silica by plants. The experimentally established fact of the absorption of SiO_2 from silica gels has also been produced as evidence in favor of the theory of colloidal penetration. The researches of Pfeffer (71), Gregoire (30), and especially of Jennings (42) leaves no room for doubt that silica when supplied either in the gel or sol condition is absorbed. Jennings used freshly prepared silica gels from sodium silicate, which were added to nutrient solutions in which wheat plants were grown. Analyses of these plants with the controls showed definite and relatively large absorption of silica.

But the conclusion that the absorption necessarily takes place in the colloidal condition *per se* is not valid. The facts, as we shall see, are also consistent with the solution theory.

(*1*) *The "solubility" of silica.* Truog (87), skeptical of the claim of the direct absorption of colloidal silica, offers as evidence against it that silica is soluble to the extent of 430 p.p.m. But difficulties at once arise with respect to the connotation "solubility." Lenher and Merrill (50) give 428 p.p.m. and Gile and Smith (27) give about 92 p.p.m. as the solubility of silica sols (silicic acid) in conductivity water. The former used S & S filter paper No. 589 and the latter Chamberlain filters. The researches of Joseph and Hancock (44) indicate a solubility of 32 p.p.m for anhydrous silica.

All these investigators are careful to qualify their use of the term "solubility" by the explanation that all or part may be present as a finely dispersed sol rather than a true solution. It is, therefore, all the more interesting to note that more than twenty years ago Mylius and Grosehuff (63) by treating sodium silicate with hydrochloric acid prepared an unstable metasilicic acid that passed through a semi-permeable membrane.

Norton and Roth (64) also conclude that silicic acid may exist in true solution. The presence of definite silicic acids, such as orthosilicic acid(H_4SiO_4), in silica hydrogels and hydrosols, having the composition $xSiO_2 \cdot yH_4SiO_4 \cdot zH_2O$, in which the quantities of water and silica might vary from a true solution of silica in water through the highly dispersed gels, was indicated.

The writer (84) found that the silica in soil solutions obtained by displacement methods (69) dialyzed through collodion membranes prepared according to the method of Brown (7). McGeorge (57), too, found dialyzable forms of silica in all the Hawaiian soils examined and "little or no colloid forms." In the writer's experiments the dialysates contained 30 to 43 p.p.m. SiO_2. Moreover, owing to the well-known effect of the coagulating effect of ions, it is difficult to understand how, in the usual procedure for the analysis of clays, the sol could possibly remain stable in the presence of such large quantities of electrolytes, if the silica is not molecularly dispersed.

It is possible to interpret these results in accordance with the view presented by Hardy (34), viz., that silica may become soluble by the splitting off of small units of the adsorption complex, $xSiO_2 \cdot yH_2O$, owing to the ability of the "combined" water molecules to ionize.

The existence of pure silica hydrosols and hydrogels, as already mentioned, has been questioned. Hardy (34) concludes that the weight of evidence favors the view that hydrous silica is an ampholyte possessing an isoelectric point well on the acid side (pH 6.4), and records Williams' conclusions (95) that silica gel seems to possess a reticulate structure which when saturated contains water in two phases, the one absorbed at the surface of the material comprising the gel framework and the other filling the intermicellar spaces.

From the facts presented above, it would be unscientific to infer that because an increased absorption of silica by plants is found when silica gels are added to nutrient solutions absorption of the colloid takes place *per se*. The facts are obviously equally consistent with the simpler view of absorption from true solution.

(*2*) *The effect of silica gels on the absorption of other ions by plants.* Finally, it is of interest to inquire into the causes producing an increased absorption of phosphorus from rock phosphate in the presence of silica gels. Lemmermann and Weismann (49) attributed the increased yields to an increased assimilation of silica. This view was criticized by Gile and Smith (27), who contended that the beneficial action of silica gel on the growth of plants is to be attributed to the effect of silica on the solubility of rock phosphate. There is yet another possible explanation, viz., that derived from the application of the Gibbs-Donnan equilibrium law to the distribution of ions on the two sides of the semi-permeable membrane. This latter view is supported by the work of Butkewitsch and Butkewitsch (9), who have supplied experimental evidence on the influence of the presence of a non-diffusible ion (silica) upon the absorption of diffusible ions by plants. The application of the Gibbs-Donnan membrane equilibrium theory will be discussed in a subsequent paper.

c. The absorption of iron by plants. Comber (14) is unable to reconcile the absorption of iron by plants on calcareous soils with the solution theory.

This view is based on the insolubility of ferric oxide. It should be noted, however, that iron may exist in soils in comparatively large amounts in the *lower state of oxidation.* Thus, Vanstone (89) records the existence of iron in soil from the Folkstone Beds of England as the soluble and available ferrous phosphate.

Owing to the weakly basic character of Fe_2O_3, the ferric carbonate formed in calcareous soils is unstable, undergoing hydrolysis with formation of the sol $xFe_2O_3 \cdot yH_2O$, or under some conditions, a precipitate of ferric hydrate (12). Hydrous ferric oxide is an amphoteric electrolyte with a pH probably of about 6.5. Its basic properties, however, outweigh its acidic properties and the "ferrites" so formed are quite unstable. Its acidic dissociation, $Fe(OH)_3 \rightarrow 3(OH)^- + Fe^{\cdot\cdot\cdot}$, therefore, would, as recorded by Hardy (34), be accompanied by the formation of the irreversible mono-hydrate. The precipitated hydroxide has a low solubility in water (1 p.p.m.); if, however, conditions permitted the rate of absorption even at this low concentration to be high, enough Fe might still be furnished for normal metabolism by the disturbance of equilibrium, as already discussed (p. 253). The investigations of Gile and Carrero (25), who showed that soils which yield iron sufficient for the growth of plants may not show a detectable amount of iron in the water extract, may be cited in support of this view. The experimental evidence, without exception, in culture solutions has shown that only traces of iron need be present in the solution for normal growth.

If, on the other hand, Joffe and McLean's conclusions (43) from laboratory experiments are applicable to soil conditions, then we must infer that iron cannot occur in molecularly dispersed solutions in normal soils and very little in the sol state, since no $Fe(OH)_3$ in molecularly dispersed solution was found by them higher than pH 3 to 4; and that even in the presence of SO_4 ions no $Fe(OH)_3$ sol existed above pH 3.8. Apparently, however, in the presence of NO_3 and Cl ions, the $Fe(OH)_3$ sol could exist as high as pH 5.4. These results indicate the importance of the consideration of the influence of the other ions in the soil.

Since the criterion of the sol condition used by Joffe and McLean was non-diffusibility through collodion sacs, this criterion, as was indicated in the earlier part of this paper, may invalidate the sharp line of distinction made by these authors between true solution and the sol and gel conditions.

It would be of interest to correlate Joffe and McLean's results with Gile and Carrero's culture experiments (26), in which these authors show that colloidal iron that was not permeable to a parchment membrane was available to plants in an acid medium and to a limited extent in a neutral medium but not when $CaCO_3$ was added. Unfortunately, Gile and Carrero do not give the pH of their solutions.

In an earlier paper (25) Gile and Carrero, comparing the availability of dialyzed iron with ferric chloride to rice plants in culture solutions, conclude that rice could not assimilate "colloidal iron" but that the plants absorbed the fraction of the iron that was molecularly dispersed from the dialyzed ferric oxide sol.

Hardy (35) believes that complex coördinated metal anions may readily penetrate and suffer translocation in the plant. This belief is founded on the results obtained by many investigators who have used different iron compounds in nutrient culture experiments; but, as there is no definite information on the composition of these complex organo-metallic compounds (36), the assumption that they are absorbed by plants seems unwarranted. There can be little doubt that the complex ferro-citrates and ferro-tartrates, as obtained by extracting iron oxides with citric and tartaric acids, are colloidal and that on standing they gradually undergo hydrolysis and precipitate out; but there is still some ferric ion in solution even after 60 days (36). In this connection it is interesting to note that analysis by the writer of a sample of Merck's "dialyzed iron containing 5 per cent Fe_2O_3" gave, after coagulating with $MgCl_2$ and filtering, 0.5 per cent of chlorine. Tested with potassium sulphocyanate the filtrate gave a slight blood red color. The presence, therefore, of some Fe in solution is indicated. It follows that the colloid-solution equilibrium hypothesis advanced earlier in this paper (p. 253) may also be applied to explain the penetration of iron (and aluminum) into cells. According to this view we would have:

$$\text{Colloidal } Fe_2O_3 \cdot xH_2O \rightleftarrows \text{dissolved } Fe(OH)_3 \rightleftarrows Fe^{\cdot\cdot\cdot} + 3(OH)^-$$

Relative to the absorption of other ferric sols by plants the experiments of Parker (70) show that colloidal ferric phosphate hydrosol, that was non-diffusible through collodion sacs, was not available to plants.

Field experiments on the subject show strange contradictions. Thus, Gile and Carrero's field experiments on calcareous soils indicate that iron-induced chlorosis may result in plants growing upon them. We are, therefore, on the horns of a dilemma. Iron is available to plants on the chalk soils of England, but in the calcareous soils of Hawaii it is not available in sufficient amounts to prevent chlorosis. The results on the Hawaiian soils do not support the explanation offered (14, 35) for the absorption of iron, viz., the formation of *organo-compounds* with such substances as sugars, starches, certain proteins, etc.

The conclusion seems inevitable, therefore, that more facts are needed before any claim to the absorption of colloidally dispersed iron by plants can be made. All the facts can readily be explained on the basis of the iron of soils being absorbed in the molecularly dispersed state.

d. The availability of mineral phosphates. If the phosphorus applied both in the "raw" mineral form and as superphosphate is present in the colloidally dispersed phase, it would not be possible, as many investigators have done, to find a correlation between the amount of water-soluble phosphorus, the amount of phosphate applied and the response of crops. In what follows, we shall discuss some of the salient points with reference to the conditions rendering the phosphorus of mineral phosphates available.

(1) *Superphosphates.* In a very ingenious discussion of the availability of phosphorus, Murray (62) points out that the compounds into which the soluble phosphates are converted by reversion in the soil will consist of particles of different sizes; some, therefore, will have larger and some smaller *surfaces.* As the particles below the critical stage (circ. 200$\mu\mu$) representing the transition to the colloidal state will undergo comparatively rapid solution, it follows that the rate of absorption of ions by the plant will be determined by the number of particles below the critical stage.

Murray cites the law of diminishing returns, which Mitscherlich and Baule (60) have attemped to express as a fundamental law, in support of this view. Thus, when the particles below the critical stage are formed in such large numbers that equilibrium exists between the rate of absorption by the plant and the rate of solution, no appreciable difference in the yield would be expected to occur by the addition of more fertilizers. This is what happens in most cases, although exceptions to the general rule are known (48).

Murray's views on the formation of solid particles are based on the hydrolysis hypothesis. The investigations of Cameron and Bell (10) lead to the conclusion that the addition of mono-calcium phosphate to the soil results in the formation of the secondary salt $CaHPO_4 \cdot 2H_2O$.

$$CaH_4(PO_4)_2 \cdot H_2O + H_2O = CaHPO_4 \cdot 2H_2O + H_3PO_4.$$

They concluded that no definite compound existed intermediate between $CaHPO_4 \cdot 2H_2O$ and $Ca(OH)_2$, but only a series of solid solutions. Austin's (1) results on the electrometric titration of $Ca(OH)_2$ and of $CaCO_3$ with mono-calcium phosphate are essentially in accord with the views of Cameron and Bell. Both Warington's (91) and Bassett's (2) investigations, however, indicate that only the tricalcic phosphate $Ca_3(PO_4)_2$ and the hydroxy-apatite $Ca_3(PO_4)_2 \cdot Ca(OH)_2$ can be in stable equilibrium with an aqueous solution, and that the latter is the only calcium phosphate that can permanently exist under normal soil conditions. Which of the foregoing conclusions apply to the actual conditions in the soil, it is impossible to say. All that is certain is that calcium mono-phosphate is converted into a substance (or substances) that is very much less soluble than the original substance and that, possibly, the conversion may be into very insoluble forms. Murray's deductions are, therefore, in no way irrational. But the actual conditions existing in the soil may not be capable of explanation wholly by solution forces, as he supposes; for, apart from the possibility of the formation of aluminum and iron phosphates in acid soils, there are unquestionably other forces operative, in addition to those pointed out on page 263, to disturb the equilibrium conditions, such as those due to absorption of the PO_4 ions by the soil colloidal complexes. The tenacity with which these absorbed *ions* will be held will vary with the potential differences developed and, therefore, upon other cations and anions present in the absorption complex (16, 79, 81). The concentration of the PO_4 on the colloidal surfaces will be determined by the Gibbs-Donnan equilibrium law.

The experimental work in support of these facts is considerable, of which some only will be cited: (*a*) The PO_4 ions of superphosphate may be so strongly absorbed by some soils that acids may not dissolve more phosphorus from such soils treated with superphosphates than from soils supplied with rock phosphate (98). (*b*) In field experiments it frequently happens that as much as 20 per cent of the applied phosphorus is not recovered by the crop (27). Wheeler and Adams (93) concluded from their experiments on peat and muck soils that "saturation" of the soil colloids was necessary before additions of phosphates became effective.

Gordon and Starkey (28, 80), working with artificial iron and aluminum gels, have shown, as would be expected from theoretical considerations, that the greater the hydrogen-ion concentration of the surrounding solution, the lower the absorption of PO_4 ions and vice versa. Nevertheless, leaching of phosphorus may occur. A slight loss of phosphorus from a loam soil derived from Triassic Red Shale has been noted in the New Jersey Station cylinder experiments (5), especially in the larger phosphatic applications. On some North Wales shaly loams and even in some heavy clay loams Robinson and Jones (75) found considerable leaching of phosphorus—applied as slag—into levels below 18 inches, in a region where the leaching of the soil by percolating waters is above normal.

The foregoing evidence on superphosphate may be insufficient to provide rigid proof that the facts are best in harmony with the solution theory; but, on the other hand, no experimental work on superphosphates has, thus far, furnished any support for the theory of the absorption of colloids. If rigid proof were forthcoming that silica gels or sols are directly absorbed by plants, then, since it forms the greater portion of the outer coating of the soil colloidal particles, phosphorus and other ions absorbed by this hydrogel might be conceived to be carried into the root hairs with the silica, but until additional facts are obtained the view must be held to be purely speculative.

(2) *Sparingly soluble phosphates.* Is it valid to deduce from the fact that fertilizers, like basic slags and mineral phosphates, are "sparingly" soluble that solubility is not the *dominating* condition of availability, as Comber (14) suggests? The terms "sparingly soluble" and "valuable" applied to such fertilizers are purely relative. On some soils and for some plants, especially the *solanaceae*, as will be discussed in a subsequent paper, the mineral phosphates may be quite unavailable. Under favorable conditions, however, in some soils the mineral phosphates, especially "floats" (raw rock phosphate ground finer than 200 mesh), may be as available as the superphosphates.

(*a*) *Solubility and availability of "floats."* Due to their variable content of $CaCO_3$ there is considerable variation in the solubility of the natural rock phosphates in water, carbonic and citric acids, etc. The extensive bibliography on the subject is recorded by Cameron and Hurst (11). These latter investigators arrived at the conclusion that rock phosphate undergoes hydrolysis as follows:

$$Ca_3(PO_4)_2 + 4H_2O = CaHPO_4 + 2CaO \cdot H_2O + H_3PO_4$$

The "solvation" of this mineral by water is, therefore, a solution not of the tertiary phosphate but of its decomposition products. It is then these secondary products that are subjected to the action of the solvent forces of the soil solution. Moreover, the solubility of these products is related to their availability. Thus, Vanstone (88) has found that all the phosphates of calcium examined by him were practically completely soluble in 2 per cent oxalic acid in half an hour at ordinary temperatures and that the intake of phosphorus by the bean plant is proportional to the oxalic acid solubility.

(*b*) *Acids as a factor.* There is some evidence to indicate that acids, especially carbon dioxide, resulting from the decomposition of organic matter may, under some soil conditions, be an important factor. The laboratory experiments of Truog (86) and the field and laboratory work of Breazeale and Burgess (6) may be cited in this connection. The latter investigators found that in the case of wheat plants the relative absorption of PO_4 from rock phosphate in soil culture solutions, treated with "floats" contained in distilled water only, was only 0.0104 gm. PO_4, but 0.0188 gm. PO_4 when the solutions were saturated with CO_2 and 0.0206 gm. PO_4 from a dilute aqueous solution of $Ca(H_2PO_4)_2$. The availability of the rock phosphate used by these investigators in soil saturated with CO_2, therefore, is not much less than that of superphosphate. Although, as will be shown in a later paper, it is unlikely that the soil solution is saturated with CO_2, nevertheless, barring secondary reactions, the marked influence of this solvent on the availability of "floats" is indicated. It is probable that the reason why the earlier investigators failed to determine the influence of the CO_2 factor on the *availability* of rock phosphate was due not only to the reasons cited by Truog (86) and to reasons mentioned later (p. 263), but also to the unfortunate selection of the solvent used in extracting the phosphoric acid. When the extraction is carried out by means of the acids (citric, nitric) used by the earlier investigators, a reverse reaction sets in between the soluble calcium salt formed and the phosphoric acid that has become soluble, resulting in the precipitation of the phosphate (88).

From the foregoing it follows that an exact formula for the solubility of rock phosphate is not possible. It may be signified by the expression $s = \int \frac{H^+}{Ca^{++}}$, indicating that the solubility increases with the hydrogen-ion concentration of the solution. We should expect, therefore, as indeed Gedroiz (24) and McCall (56) and others have already shown, an increased availability of these naturally occurring phosphates on unsaturated soils. It has already been mentioned that Vanstone (88) found that the solubility of rock phosphate in 2 per cent oxalic acid corresponded with the rate at which mineral phosphates are absorbed by plants and, furthermore, Kelly's (47) laboratory experiments have indicated a depression in the solubility of $Ca_3(PO_4)_2$ in the presence of $CaCO_3$.

However, the solution of the problem of the availability of rock phosphates to plants is not so simple as might be inferred from solubility results. The favorable factors leading to the solution of rock phosphate in the soil may be opposed by secondary reactions that tend to the "fixation" by bacteria (85) and to the precipitation by the hydrolyzing sol exchange bases, e.g., Al, Fe, (79) of the phosphorus thus rendered soluble. The new series of fertilizer plot experiments of the Pennsylvania Experiment Station, which were commenced in 1920, may be cited in illustration. In these experiments the additions of superphosphate and rock phosphate, respectively, to the *lime with manure* plots (pH 6.8) have given only approximately 15 per cent higher yields of corn, oats, and wheat than on the *lime without manure* plots (pH 5.9 to 6.0).

Current explanations, therefore, relative to the results obtained on the neutral or slightly acid soils of some of the eastern and middle western experiment stations, supplied with sufficient rainfall, to the effect that the amount of decomposing organic matter is the most important factor conditioning availability of rock phosphate, require qualification. Generalization of the explanations presented by Breazeale and Burgess (6) of their results obtained on the Western alkaline soils which they maintain contain no free CO_2 and which give no response to rock phosphate additions is, therefore, limited by other factors.

In many cases conclusions as to the relative availability of rock phosphate (and superphosphate) have been based on growth in sand culture experiments using mineral nutrient solutions. Such experiments, obviously, do not show the relative availabilities under field conditions.

There is evidence that the mineral acids and possibly also acetic, butyric and lactic acids formed in the soil increase the availability of sparingly soluble phosphates. This is supported by the work of Hopkins and Whiting (40) on the availability of rock phosphates in the presence of ammonium salts, and also by the Lipman process (53, 54) of rendering floats available by utilizing the oxidation of sulfur by micro-organisms.

(*c*) *Absorption of PO_4 from very low concentrations.* The concentration of phosphate ions in all soils is low. Numerous investigators, working with culture solutions, have shown that plants can develop normally in very dilute solutions of PO_4. Thus, Breazeale and Burgess (6) found that oat and barley seedlings can absorb PO_4 sufficient for normal development in a nutrient solution containing only 0.50 p.p.m.; and Parker (70) found that corn willl make maximum growth in culture solution containing only 0.25 p.p.m. PO_4. If these results are applicable to field conditions, the small solubility of rock phosphate would present no difficulties to the acceptance of the older solution theories.

Comber (15) points out that the naturally occurring phosphates do not seem to possess colloidal surfaces. But even if such a phosphate as "floats" was prepared in the colloidal condition and applied to the soil, it is questionable if carbon dioxide and the other solvents present would react with the colloid, owing to the influence of the electric charge carried by it.

The evidence presented affords no indication that the availability of rock phosphates is determined by any colloidal behavior. Rather does it point toward the conclusion that its availability is a question of absorption from solution as determined by the laws of diffusion and mass action, through the operation of which the rate of absorption of PO_4 ions by the plant is a function of the rate of solution of the mineral phosphate.

The behavior of other minerals is similar. Thus, the potassium or orthoclase has a solubility of 282 p.p.m. in distilled water. One could hardly, therefore, attribute the results obtained by Haley (32), in which 200-mesh orthoclase used in sand cultures furnished potassium at a rate ample to produce large yields of buckwheat, to the colloidal condition of the orthoclase.

(*d*) *Iron and aluminum phosphates.* Although there is evidence (58) to indicate that the *artificially prepared* phosphatic salts of iron and aluminum are more available to plants than the *naturally occurring* phosphates of these metals, there is nothing to indicate that colloidal behavior is the cause of this difference. Unfortunately, Marais (58) does not give the source of his "C.P." materials, nor the relative fineness of division of any of the phosphates used. Although it is possible that the greater fineness of division of the artificial phosphates as well as the tendency towards increasing basicity and consequent insolubility of the natural products, as weathering proceeds, may be contributary causes, other factors may predominate. Thus, in the case of artificially prepared ferric phosphate, for example, Cameron and Bell (10) have shown that the acidic properties of the salt prepared by precipitation may be very pronounced, even after 100 washings, due to absorbed PO_4 ions. The salt thus prepared is more soluble and, therefore, would be more available than the pure product.

Moreover, since Marais (58) did not find the *mineral* aluminum and iron phosphates of any value except in the presence of ammonium compounds, it would be logical to infer that the availability of these mineral phosphates in the presence of nitrogenous salts would be due to the action of the nitric acid formed (40), the rate of solution being determined by the number of particles below the critical stage (p. 260). However, it must be admitted that our knowledge of the conditions rendering the various phosphates in the soil soluble are very unsatisfactory. Over twenty years ago it was suggested by Whitson and Stoddart (94) that the reason why acid soils act as if deficient in phosphorus is due to the formation of aluminum (and iron) salts. Vanstone's (89) experiments on the solubility of soils in citric acid give confirmative evidence of the relative insolubility of the phosphorus of acid soils. But the findings of some investigators (58, 93) that the availability and, therefore, presumably the solubility of aluminum phosphate is increased by liming is not explicable on theoretical grounds. The equilibrium conditions of a solution involving Al (or Fe), Ca, PO_4, H and OH ions may be written

$$AlPO_4 \rightleftarrows Al^{+++} + PO_4^{---} \tag{1}$$
$$3Ca(OH)_2 \rightleftarrows 6OH^- + 3Ca^{++} \tag{2}$$

$$Al^{+++} + 6OH^- \rightleftarrows Al(OH)_3 \tag{3}$$
$$PO_4^{---} + 3Ca^{++} \rightleftarrows Ca_3(PO_4)_2 \tag{4}$$

As the solubility product of $AlPO_4$ is less than $Ca_3(PO_4)_2$, most of the PO_4 ions, at pH 7.0 and above, would be precipitated as $AlPO_4$ (or $FePO_4$). The tendency of lime would, therefore, be to depress the solubility of aluminum and ferric phosphates.

Teakle (81) suggests from the results of his experiments that the following reactions may take place.

$$AlPO_4 + 6OH^- \underset{\text{below pH 6.4}}{\overset{\text{above pH 6.4}}{\rightleftarrows}} AlO_3 + PO_4^- + 3H_2O \tag{1}$$

and

$$FePO_4 + 3OH^- \underset{\text{below pH 3.0}}{\overset{\text{above pH 3.0}}{\rightleftarrows}} Fe(OH)_3 + PO_4^- \tag{2}$$

also

$$Ca_3(PO_4)_2 + 6OH^- \xleftarrow{\text{neutral conditions}} Ca(OH)_2 + 2PO_4^- \tag{3}$$

$$x + \frac{y}{3}\,Ca_3(PO_4)_2 + 2y(OH)^- \xrightarrow[\text{alkaline conditions}]{} xCa_3(PO_4)_2 \cdot yCa(OH)_2 + \frac{2y}{3}\,PO_4^- \tag{4}$$

Under more alkaline conditions the calcic phosphate would give rise to a basic phosphate and PO_4 ions. But considerations of the solubility products of these substances lead to difficulties in accepting Teakle's conclusions *in toto*.

Austin (1) has challenged the belief that the formation of insoluble complexes of iron and aluminum phosphate in acid soils is the cause of the low availability of phosphorus found in many acid soils. He did not, however, as the title would seem to suggest, use any soils in his experiments. The data was obtained by working with the pure hydrosol or hydrogel bases assumed to be present in soils, by titrating electrometrically with $CaH_4(PO_4)_2$. Austin's conclusion is based on the fact that the titration of $Al(OH)_3$ and $Fe(OH)_3$ with $CaH_4(PO_4)_2$ resulted in 82 per cent and 40 per cent, respectively, of the phosphorus still being in solution at pH 5.4, from which fact he concludes that at pH 5.4 the equations

$$Al(OH)_3 + CaH_4(PO_4)_2 \rightarrow AlPO_4 + CaHPO_4 + 3H_2O$$
$$Fe(OH)_3 + CaH_4(PO_4)_2 \rightarrow FePO_4 + CaHPO_4 + 3H_2O$$

do not go to completion. In other words, in the presence of Al (or Fe), Ca, PO_4, H and OH ions a fairly acid soil would not have all the PO_4 ions combined with Al (or Fe).

The actual conditions in the soil are undoubtedly complex. Hydrous alumina has an isoelectric point of 6.5. If we consider its acidic dissociation

$Al(OH)_3 \rightarrow Al^{\cdots} + 3OH^-$ obviously the addition of an acid will result in the formation of water by the combination of the $H^{\cdot}$ of the acid and OH^- of the alumina. It follows, as Hardy (34) has indicated, that the colloidal hydrous alumina would be removed further from its isoelectric point and the potential difference between its surface and the dispersion medium would be increased. The addition of an anion, such as PO_4 or SiO_2, would—depending on the other anions and cations present—result in the formation of insoluble complexes. The precipitating power of other anions would depend on their relative electro-negative affinity. As the soil solution became more acid we should expect Al (and Fe) ions to come into solution, for both aluminum phosphate and ferric phosphate become more soluble the higher the hydrogen-ion concentration of the medium. The system resulting would depend on the solubility products of the substances formed.

(*e*) *Basic slags*. The expression "insoluble but available to plants" by which clays have been characterized does not, without qualification, appear justifiable. The old Thomas slag obtained by the Thomas-Gilchrist-Bessemer process, which was produced by the addition of a basic "liming" of $CaCO_3$ and $MgCO_3$, was of a fairly constant composition, the phosphorus of which is from 80 to 95 per cent available to plants and showed close correlation with solubility in 2 per cent citric acid. The first "run off" used for the agricultural slag has a high phosphorus content. This Gilchrist-Bessemer process, however, has now, for economical reasons, been largely superceded in Europe by the Siemens-Martin process, in which a calcium fluoride flux is used, resulting in slags of very variable composition and phosphorus availability (6 to 50 per cent), but which show little correlation with solubility in 2 per cent citric acid. The slags from the acid-Bessemer and Basic Open Hearth processes used in the United States are not high in phosphorus and, moreover, the ores used for the making of steel in this country are invariably low in phosphorus.

Stead, as recorded by Vanstone (90), gives $Ca_3(PO_4)_2 \cdot CaO \cdot CaSiO_3$ as the composition of the low acid soluble slags. The most soluble phosphate has the composition $5CaO \cdot SiO_2 \cdot P_2O_5$. When these silico-phosphates are applied to the soil the combined CaO is freed as weathering proceeds, permitting the ready dissolution of the P_2O_5 radical. The period of time for this weathering process to occur and, therefore, the availability varies with the nature of the slag. The smaller the amount of silicate in the slag the higher the availability.

The behavior of slags in the soil, therefore, does not tend, without further experimental evidence, to support any theory of colloidal absorption by plants.

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BULLETIN 240 APRIL, 1929

Results of Tobacco Experiments in Pennsylvania

1922 to 1927

Tobacco Plots at Ephrata, Lancaster County, Pennsylvania

The Pennsylvania State College
SCHOOL OF AGRICULTURE AND EXPERIMENT STATION
State College, Pennsylvania

THE PENNSYLVANIA STATE COLLEGE

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*On leave of absence.

†In cooperation with the United States Department of Agriculture.

The Bulletins of the Station will be mailed regularly, free of charge, to all residents of the State who request them. Address: Director of Experiment Station, State College, Centre County, Pennsylvania.

Results of Tobacco Experiments in Pennsylvania 1922 to 1927[1]

OTTO OLSON [2] AND D. E. HALEY

THE TOBACCO investigations in Pennsylvania are conducted by the Pennsylvania Agricultural Experiment Station in cooperation with the Office of Tobacco and Plant Nutrition, Bureau of Plant Industry, United States Department of Agriculture, and the Tobacco Growers' Associations of Lancaster, Clinton, and Lycoming counties. The main objects of this work are to improve the quality and increase the yield of tobacco produced in Pennsylvania. These include a number of problems in the culture, subsequent handling and manufacture of cigar-leaf tobacco which are of major importance from the standpoint of the cigar manufacturer as well as the grower. The recent phenomenal increase in cigarette production is one of the causes for the increased interest shown lately in cigar-leaf tobacco experiments; both growers and cigar manufacturers realize the necessity of important changes in production in order to stimulate consumption.

Earlier experiments emphasized the importance of a high yield but, at present, high quality is considered to be of far greater value, par-

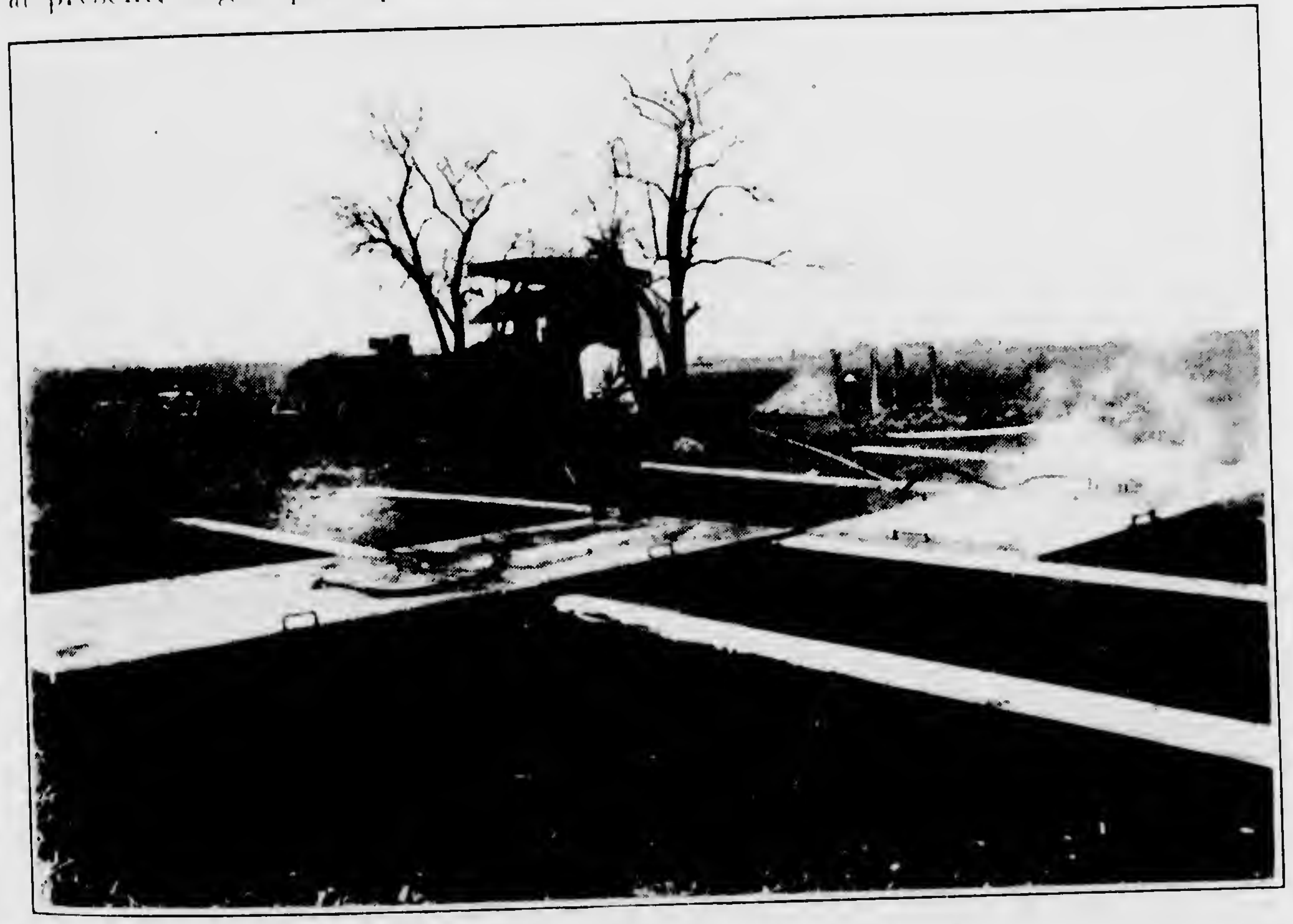

FIG. 1. STEAM STERILIZATION OF SEEDBEDS

Four pans are used. After two of them have been steamed for 40 minutes, the connections are moved to the other two pans.

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[1] The Pennsylvania Agricultural Experiment Station and the Bureau of Plant Industry, United States Department of Agriculture, cooperating.

[2] Associate Agronomist, Tobacco and Plant Nutrition Investigations, United States Department of Agriculture.

ticularly burn, flavor and aroma, which are the three most desirable qualities of cigar-leaf tobacco.

Experiments on tobacco in Lancaster county, Pennsylvania, were begun in 1893 on a comparatively small scale. In 1912 the work was increased in scope and extended to other tobacco-producing counties. Practically every phase of tobacco culture and handling has been subjected to research, including shelter tent experiments, studies on cost of production, tobacco bulk sweating, steam sterilization of seedbeds (Fig. 1), fertilizer experiments on seedbeds, rate of seeding (Fig. 2), fumigation of seedbeds, seed cleaning and grading, fertilizer experiments in several counties, planting distances and topping heights of plants in the field, topping, suckering, root rot of tobacco, strain tests of the varieties produced, variety tests, seed breeding (Fig. 3), reducing the nicotine content of tobacco, increasing the nicotine content of tobacco, curing tobacco with the use of artificial heat in order to prevent pole-burn, and re-drying the cured tobacco to prevent black rot. Detailed descriptions of most of these experiments are available in various annual reports and bulletins published by the Pennsylvania Agricultural Experiment Station.

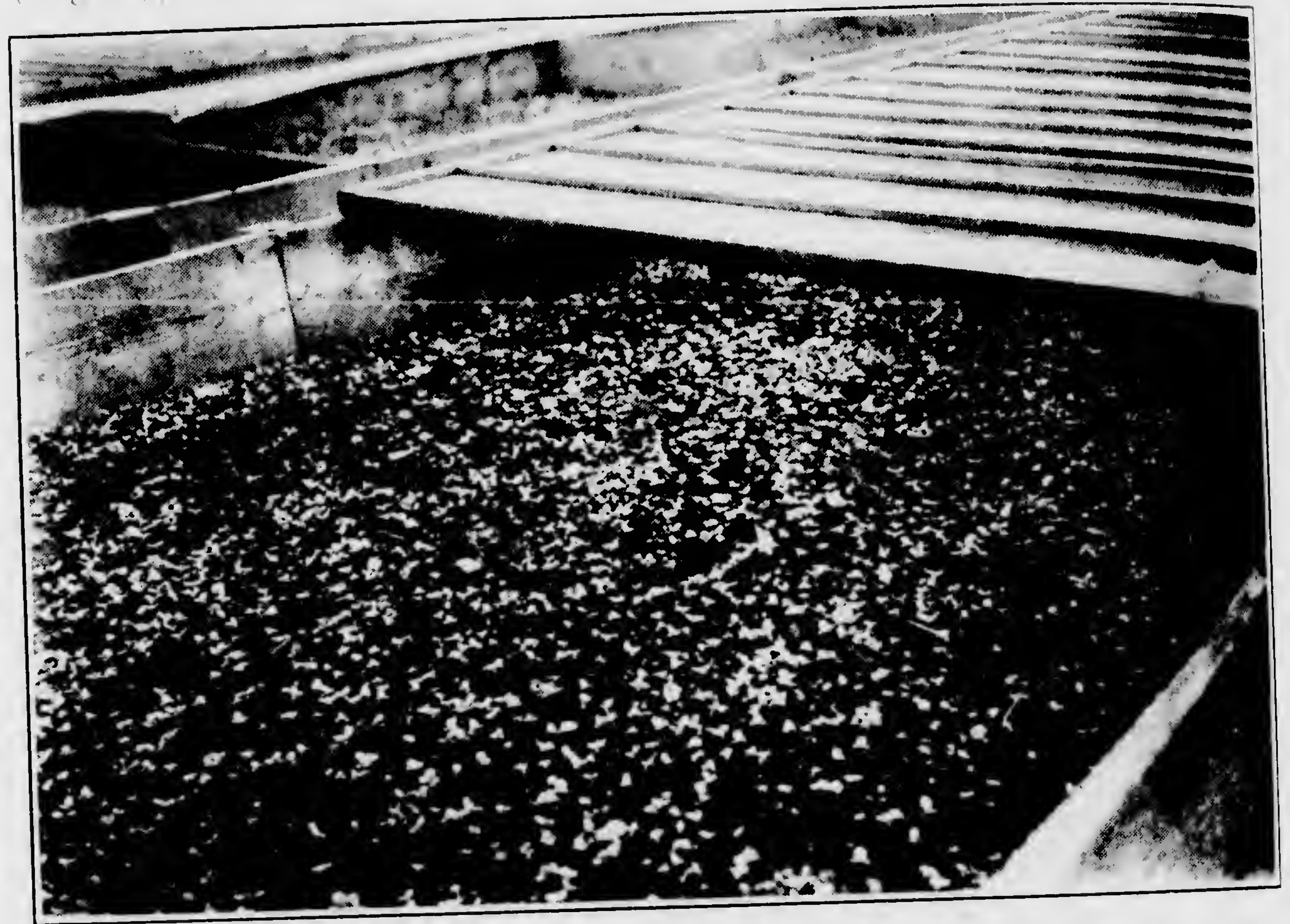

FIG. 2. TOBACCO SEEDBEDS, SHOWING A STAND OF ABOUT FOUR PLANTS TO THE SQUARE INCH, OBTAINED BY APPLYING SEEDS AT THE RATE OF ONE OUNCE PER 800 SQUARE FEET

Fertilizer Experiments at Ephrata

Fertilizer experiments in Lancaster county had been conducted on the same field for years. While the yield and quality did not show any deterioration, it was felt that there should be a definite rotation, in conformity with the farming practice in this locality. Hence, additional land was secured near the town of Ephrata, and a field of three acres was divided into three plots of one acre each, for a rotation of tobacco, wheat and clover, and alfalfa. The work was conducted on duplicate plots during 1922, 1923, and 1924. The results are given in Table 1.

FIG. 3. BREEDING PLOTS AT EPHRATA, SHOWING THE FOURTH GENERATION OF A PROMISING NEW CROSS BETWEEN THE SLAUGHTER AND HIRSHMAN STRAINS, "PENNSYLVANIA SEEDLEAF," WHICH IS RESISTANT TO ROOT ROT

These results confirm previous experimental evidence; they show, beyond reasonable doubt, that a high-grade commercial fertilizer pays. This is strikingly shown in the yields for 1923, when a severe drought occurred during the growing season. It is generally believed by tobacco growers that any commercial fertilizer is worse than useless in a dry season, but the records for 1923 show that an increased yield, ranging from 50 to 200 pounds per acre, was obtained by the use of commercial fertilizer. Moreover, the burn, aroma, and flavor were also improved.

Although fertilizer experiments on cigar-filler tobacco have been conducted for a number of years on the Ephrata plots, very little effort has been made to study the effect of the fertilizer combinations upon the quality of the leaf, partly because of lack of funds and partly because of lack of interest among growers and packers, so that practically the sole object of these experiments in the past has been to increase the yield per acre. During the latter part of 1924, however, a definite de-ticularly burn, flavor and aroma, which are the three most desirable qualities of cigar-leaf tobacco.

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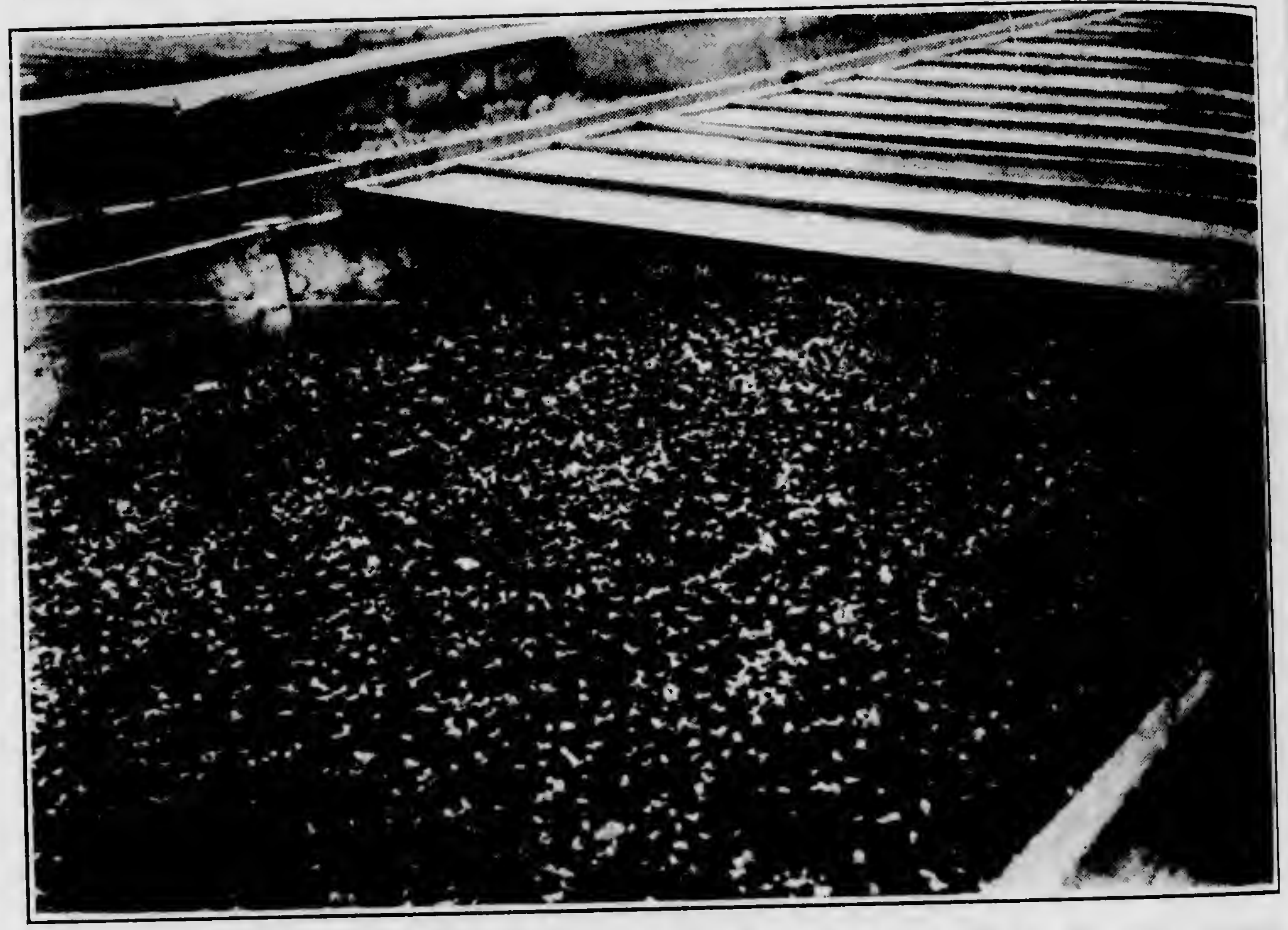
Fig. 2. Tobacco Seedbeds, Showing a Stand of About Four Plants to the Square Inch, Obtained by Applying Seeds at the Rate of One Ounce Per 800 Square Feet

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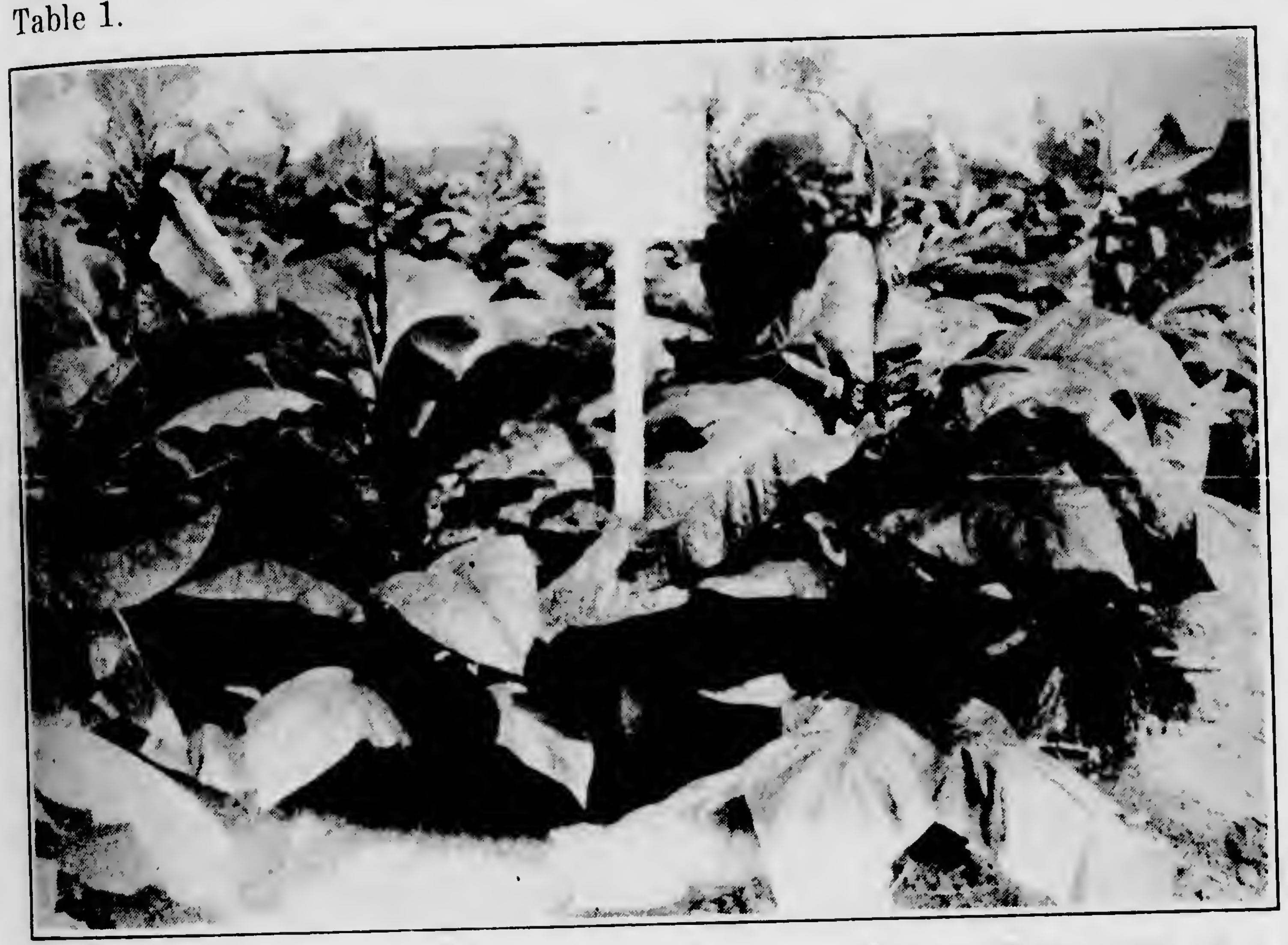
Fig. 3. Breeding Plots at Ephrata, Showing the Fourth Generation of a Promising New Cross Between the Slaughter and Hirshman Strains, "Pennsylvania Seedleaf," Which is Resistant to Root Rot

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INTENTIONAL 2ND EXPOSURE

TABLE 1. THE YIELD OF TOBACCO AS INFLUENCED BY FERTILIZER TREATMENT AND SEASON

Plot	Acre treatment	Yield per acre			
		Year 1922	Year 1923	Year 1924	Yearly average
		pounds	pounds	pounds	pounds
1	20,000 pounds of stable manure	1638	1320	1727	1560
2	20,000 pounds manure—300 pounds superphosphate (16% P_2O_5), and 200 pounds sulphate of potash	1833	1478	1936	1750
3	20,000 pounds manure—300 pounds superphosphate—400 pounds sulphate of potash and magnesia	1859	1477	1957	1764
4	20,000 pounds manure—500 pounds cottonseed meal—300 pounds superphosphate—200 pounds sulphate of potash	2086	1372	2200	1900
5	20,000 pounds manure—500 pounds cottonseed meal—300 pounds superphosphate—400 pounds sulphate of potash and magnesia	2052	1517	2200	1923

mand came from cigar manufacturers for better quality in cigar-leaf tobacco, as a result of economic changes in the cigar industry. The scope of the work was modified, therefore, to include a study of the following problems:

1. The effect of different fertilizer treatments on the yield and chemical composition of Pennsylvania Seedleaf or Broadleaf, Hibshman Strain.

2. The quantity of inorganic material removed by tobacco from an acre of soil.

3. The effect of field treatment on the burning quality of this tobacco; and the correlation if any, between yield, burning quality, and chemical composition.

4. The effect of fertilizer treatment on the catalase and oxidase content of tobacco.

5. The effects of the different fertilizer treatments on the composition and reaction of the soil.

6. The yield and quality of tobacco following corn, as compared with tobacco following grass and tobacco grown on the same field continuously.

In order to reach these objectives, the plans of the experimental work were changed in 1925 so as to include fertilizer experiments on a 3-year rotation system of tobacco, wheat, and grass; a 4-year rotation of tobacco, wheat, grass, and corn; and continuous cropping of tobacco on the same field. The results obtained over a 3-year period are given in Table 2.

TABLE 2. THE YIELD OF TOBACCO AS INFLUENCED BY THE FERTILIZER TREATMENT, THE SEASON, AND THE SYSTEM OF ROTATION

Plot	Rotation	Acre treatment	Yield per acre			
			Year 1925	Year 1926	Year 1927	Yearly average
			pounds	pounds	pounds	pounds
A-1	3-year	20,000 pounds manure	1593	870	1148	1207
A-2	3-year	20,000 pounds manure—400 pounds cottonseed meal—250 pounds bone flour—200 pounds muriate of potash	2079	1410	1740	1743
A-3	3-year	20,000 pounds manure—500 pounds cottonseed meal—300 pounds superphosphate—200 pounds muriate of potash	2160	1350	1008	1506
A-4	3-year	20,000 pounds manure—500 pounds cottonseed meal—300 pounds superphosphate—400 pounds manure salt	2079	1380	1540	1663
A-5	3-year	20,000 pounds manure—500 pounds cottonseed meal—300 pounds superphosphate—200 pounds sulphate of potash	2106	1410	1740	1732
A-6	3-year	20,000 pounds manure—400 pounds cottonseed meal—250 pounds bone flour—200 pounds sulphate of potash	1936	1170	1740	1612
B-1	4-year	20,000 pounds manure	1620	960	1155	1242
B-2	4-year	20,000 pounds manure—65 pounds urea—300 pounds superphosphate—200 pounds muriate of potash	1957	1425	1638	1670
B-3	4-year	20,000 pounds manure—65 pounds urea—300 pounds superphosphate—200 pounds sulphate of potash	1890	1190	1688	1590
B-4	4-year	20,000 pounds manure—214 pounds nitrate of potash—120 pounds precipitated bone	2079	1245	1600	1641
B-5	4-year	20,000 pounds manure—85 pounds ammonium nitrate—300 pounds superphosphate—200 pounds sulphate of potash	1998	1365	1646	1663
H-1	Continuous cropping	No fertilizer	1500	870	870	1080
H-2	Continuous cropping	130 pounds urea—300 pounds superphosphate—200 pounds muriate of potash	1800	1395	1470	1552
H-3	Continuous cropping	130 pounds urea—300 pounds superphosphate—200 pounds sulphate of potash	1515	1310	1250	1352
H-4	Continuous cropping	1,000 pounds cottonseed meal—300 pounds superphosphate—200 pounds sulphate of potash	1509	1320	1290	1370
H-5	Continuous cropping	428 pounds nitrate of potash—120 pounds precipitated bone	1800	1425	1545	1590
H-6	Continuous cropping	170 pounds ammonium nitrate—300 pounds superphosphate—200 pounds sulphate of potash	2025	1320	1260	1538

Severe rain storms damaged the crop badly during the three summers this experiment was in progress, especially on the field used for the continuous cropping experiment, so that the yields reported do not fully indicate the relative value of the different treatments.

Cooperative Fertilizer Experiments in Lancaster County

Tobacco growers in Lancaster county regard stable manure as essential for the production of good quality tobacco and the farming systems employed are based on winter steer-feeding for the production of a sufficient supply of this material. Stable manure, however, is not a well-balanced fertilizer, as it contains too small a proportion of phosphorus and potash to nitrogen, and it is also suspected of imparting a "strong" taste to the tobacco. At the request of cigar manufacturers, who pointed out the necessity of producing a cigar-filler tobacco of mild taste, experiments were begun to determine the possibility of growing tobacco of high quality with a fertilizer formula of high-grade materials, to be used on a 4-year rotation of tobacco, wheat, clover, and corn, the manure ordinarily used on the tobacco to be applied to corn, preceding tobacco.

The work was begun in the spring of 1926 on three farms of different soil types and was extended in 1927 to eight farms. While unfavorable weather during these two years caused considerable damage to the crops in the field and to the tobacco in the curing sheds, the results in yield and quality seem to indicate the possibility of producing cigar-filler tobacco of high quality and yield, without the use of stable manure.

The average yield in 1926, on fields fertilized with the special formula without manure, was 1800 pounds per acre; on fields fertilized with manure, 1760 pounds. In 1927, the corresponding yields were 1912 pounds and 1789 pounds, respectively. Similar experiments conducted on Havana seed tobacco in Clinton county during 1927 show an average yield of 1350 pounds per acre where this fertilizer formula was used, as compared with 1115 pounds per acre where manure alone was used.

The approximate cost of this fertilizer was $70 per ton, or $35 per acre, while the cost of 15 tons of manure—the usual application—was approximately $67.50. This gain in yield at less cost was accompanied by a distinct improvement in quality of the tobacco.

The formula which was used, 6-5-10, may be obtained by using 610 pounds cottonseed meal, 130 pounds nitrate of soda, 140 pounds precipitated bone, and 155 pounds carbonate of potash per acre.

Breeding and Strain Tests

Tobacco breeding and strain tests have been conducted for a number of years in Pennsylvania for the purpose of improving the physical characteristics of two types, namely, Seed or Broad-leaf and Havana Seed (Fig. 4). Seedleaf tobacco is used mainly for cigar fillers. Cigar manufacturers now demand not only tobacco of better quality as regards flavor, aroma, and burn, but also with a narrower leaf than is produced

at present, so that the tobacco may be stripped by machines without loss of material. Havana Seed, which is used principally for cigar binder purposes, produces a leaf which is too narrow; the trade desires a wider and somewhat shorter leaf, for economy in cigar manufacturing, while the main object of the grower is to produce a high-yielding tobacco. In order to meet these somewhat conflicting requirements, strain tests and seed-breeding have been conducted on both the Ephrata and Lock Haven plots. With the aid of the Extension Service of The Pennsylvania State College and the tobacco growers' associations in the various tobacco-producing counties, the most desirable strains have been well distributed among the growers. In 1925 to 1927, 14 strains of Broadleaf and 10 strains of Havana Seed were studied, especially in regard to habit of growth, resistance to disease, shape of leaf, and yield. Of these 24 strains, 10 were originated on the Experiment Station plots; the seeds of fourteen were collected on various farms in Lancaster, Clinton, and Tioga counties, or secured from Wisconsin, Connecticut, and New York. The results of these experiments are given in Table 3.

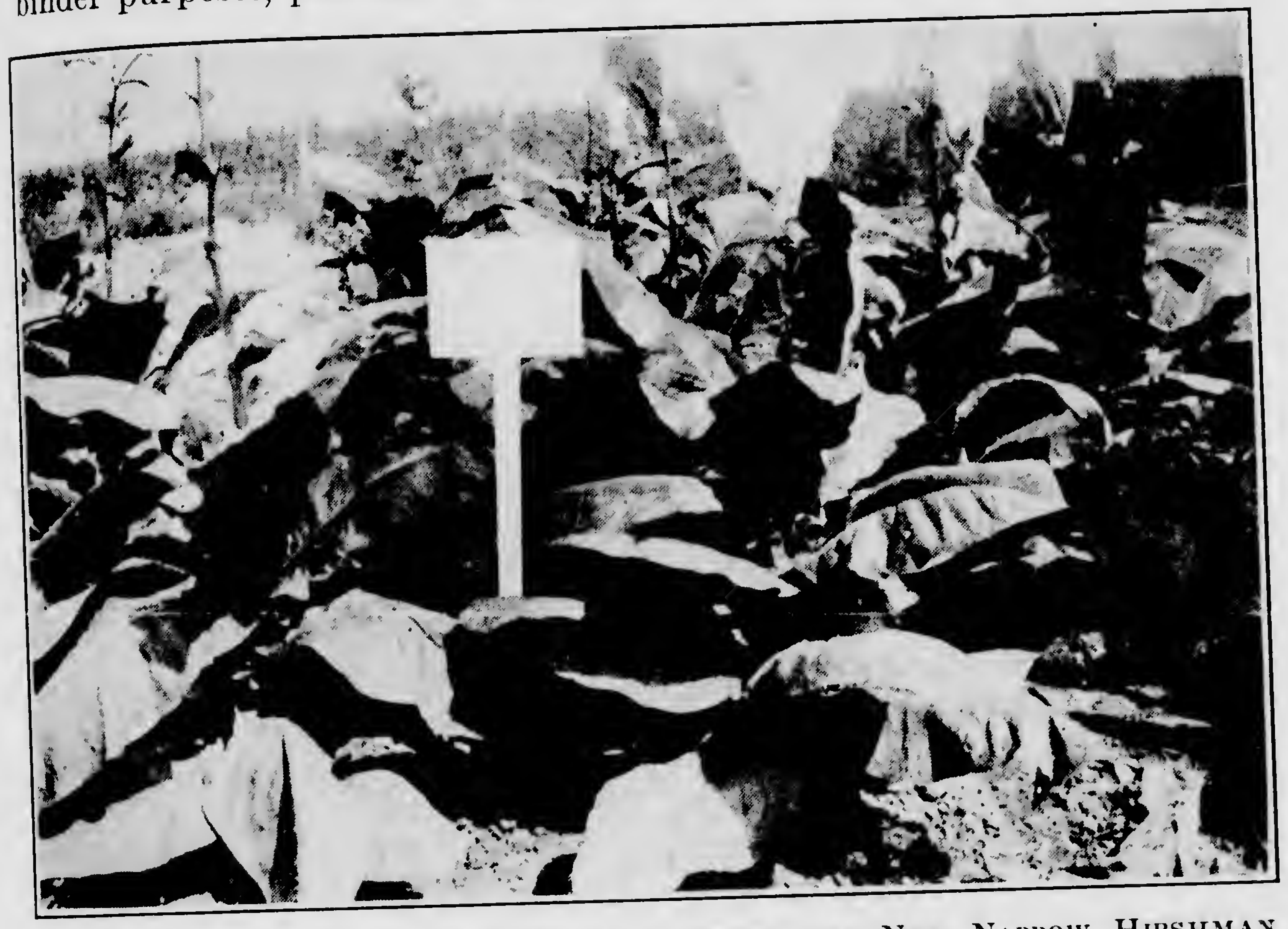

FIG. 4. BREEDING PLOTS AT EPHRATA, SHOWING THE NEW NARROW HIBSHIMAN STRAIN OF PENNSYLVANIA SEEDLEAF

The Use of Mulching Paper

During 1923 and 1924, tests were made at Ephrata to determine whether mulching paper impregnated with asphalt is of value in tobacco culture. Two plots of six rows each were used. Directly after transplanting, three rows of each plot were covered with the paper, the other

TABLE 3. THE RESULTS OF STRAIN TESTS INVOLVING RESISTANCE TO DISEASE, SHAPE OF LEAF, AND YIELD PER ACRE, FOR THE YEARS 1925, 1926 AND 1927

Strains	Origin	Resistance to disease	Shape of leaf	Yield per acre
				pounds
Broadleaf Strains:				
Pound	Lititz, Pa.	Low	Very broad	1600
Narrow Leaf	Connecticut	Low	Very narrow	1600
Glessner	Schoeneck, Pa.	Low	Very broad	1700
Fischer	Paradise, Pa.	Low	Broad	1700
Allgyer	Bird in Hand, Pa.	Fair	Fairly broad	1750
Swarr	Landisville, Pa.	Low	Rather narrow	1750
Red Rose	Intercourse, Pa.	High	Rather narrow	1800
Conn. Broadleaf Cross	Ephrata Plots	High	Broad	1750
Taylor-Hibshman Cross	Ephrata Plots	High	Broad	1800
Slaughter-Hibshman Cross	Ephrata Plots	High	Broad	1800
Slaughter	Ephrata Plots	Low	Fairly broad	1800
Hibshman-Slaughter Cross	Ephrata Plots	High	Broad	1900
Hibshman	Ephrata Plots	High	Broad	1900
Swarr-Hibshman Cross	Ephrata Plots	High	Rather narrow	1950
Havana Seed Strains:				
Reichert	York, Pa.	Low	Narrow	1150
Comstock	Lawrenceville, Pa.	Low	Narrow	1200
Conn. Havana	Connecticut	Low	Narrow	1225
Du Bon	Connecticut	Low	Narrow	1250
Pompella	Tioga, Pa.	Fair	Broad	1280
Pompella	Nelson, Pa.	Fair	Broad	1290
Resistant	Wisconsin	High	Broad	1500
Ramm	Lock Haven Plots	Fair	Narrow	1350
No. 18	Lock Haven Plots	High	Broad	1400
No. 19	Lock Haven Plots	High	Broad	1500

FIG. 5. BREEDING PLOTS AT LOCK HAVEN, SHOWING A NEW DISEASE-RESISTANT STRAIN OF PENNSYLVANIA HAVANA SEED

three rows being left uncovered. The main problem was to keep the paper from blowing away. The covered rows, of course, were not cultivated but the uncovered parts of the plots were cultivated regularly.

The growth of the plants on the paper covered rows was more rapid and, as a result, the tobacco was harvested some ten days earlier than the tobacco on the cultivated rows. Moreover, no weeds were present and the soil temperature was observed to be from 2 to 4 degrees higher than that of the uncovered plots. The treatments and yields are given in Table 4.

TABLE 4. USE OF MULCH PAPER ON TOBACCO

Fertilizer treatment	Yields per acre	
	Uncovered rows	Covered rows
	pounds	pounds
Ten tons manure per acre	1470	1785
Ten tons manure—500 pounds cottonseed meal—300 pounds superphosphate—200 pounds sulphate of potash, per acre	1710	2100

The quality of the tobacco grown on the covered rows was superior to the tobacco grown on the uncovered plots. The leaves were fairly thin and silky, these experiments covered only two seasons; further work is needed before final conclusions can be drawn. The cost of the paper, which is about $100 an acre, is probably too high for the economical production of cigar-filler tobacco.

Reducing the Nicotine Content of Cigar-leaf Tobacco

Nicotine is an alkaloid found, thus far, only in the tobacco plant. It is, probably, the chief cause of certain physiological effects of tobacco on the human system. While nicotine may be removed from tobacco wholly or in part, by chemical and physical treatments, none of the numerous attempts have been entirely satisfactory, as the flavor and aroma of the tobacco are invariably injured by even the mildest treatment. It has been shown, however, in the experiments conducted at Ephrata, that it is possible to reduce the nicotine content of tobacco by systematic breeding, although the variation in the amount in individual plants is considerable. Moreover, weather conditions, which cannot be controlled, have a marked effect upon the nicotine content.

The chief problem at present is to increase the yield per acre of the strains of low nicotine content which have been secured after several years of breeding, and at the same time hold down the nicotine content. The nearest approach to a good yield per acre has been type 73, a cross between Texas-Cuban, the nicotine content of which sometimes is as low as 0.78 per cent, and Pennsylvania Seedleaf, Slaughter Strain, which had been previously bred to as low as 2 per cent of nicotine. The nicotine content of this cross has averaged about 1¼ per cent, and the leaf yield per acre has averaged 1500 pounds.

The Production of Tobacco of High Nicotine Content

The experiments in growing tobacco of high nicotine content at Ephrata since 1915, and at Lock Haven since 1921, received considerable impetus from the discovery at The Pennsylvania State College of the high insecticidal value of dust produced from the *Nicotiana rustica* type[3]. This type of tobacco now is being cultivated and manufactured on a fairly large scale at Lock Haven, Clinton county.

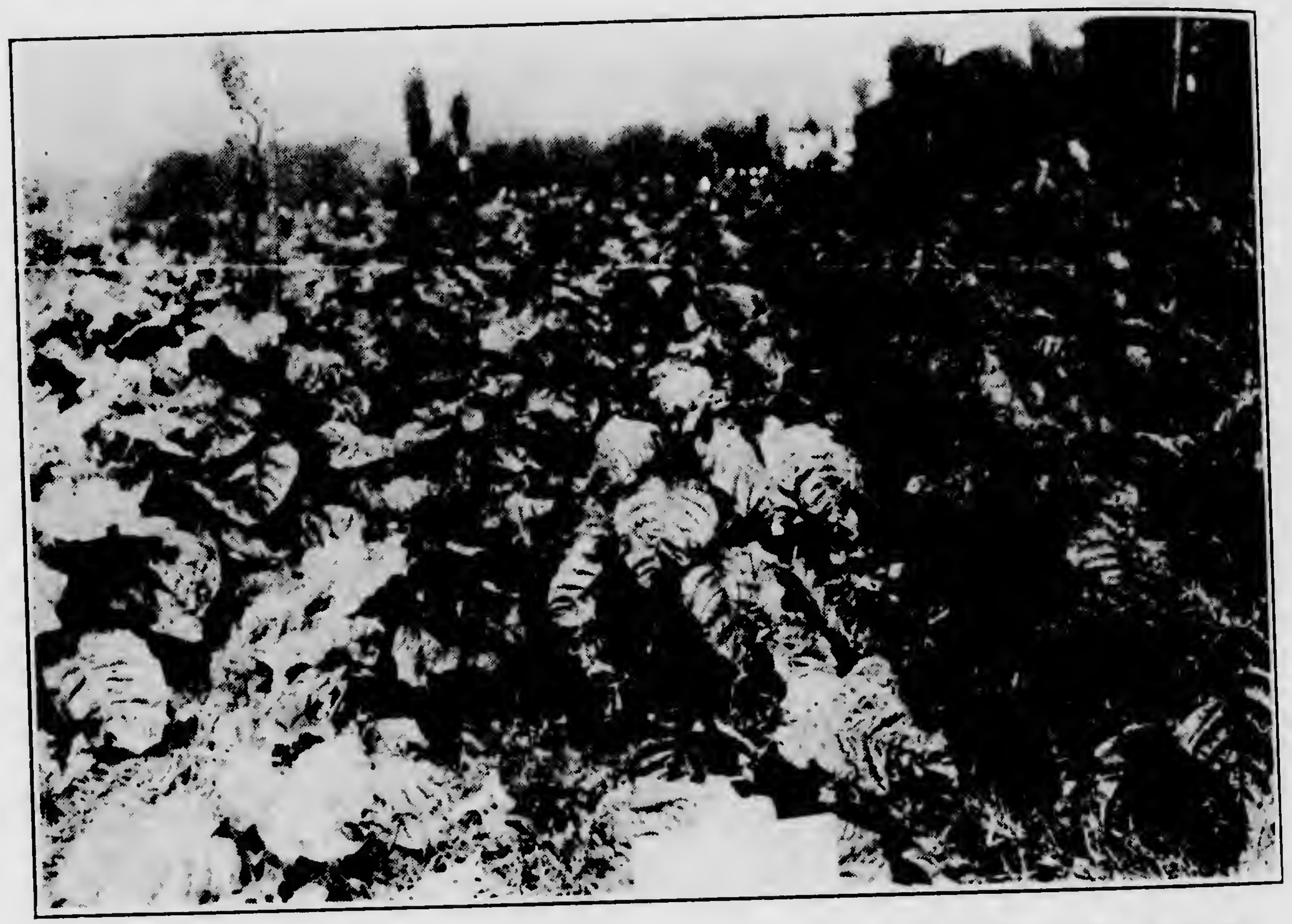

FIG. 6. BREEDING PLOTS AT LOCK HAVEN, SHOWING HIGH-NICOTINE STRAINS

The chief obstacle to successful production of *rustica* tobacco is its relatively low yield of leaf, coupled with a rather variable nicotine content. Weather conditions control the latter; it is much higher in dry than in wet seasons. It was decided to conduct fertilizer experiments on *rustica* tobacco in connection with the breeding work, in order to increase the yield of leaf per acre. These experiments were conducted at Lock Haven during 1924 and 1925; the results follow:

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[3] Haley, D. E., Gardner, F. D., and Whitney, R. T. 1924. Nicotiana rustica as a source of nicotine for insect control. Science, Vol. 60, No. 1555, p. 365.

Haley, D. E., Olson, Otto, and Follweiler, F. L. 1925. Studies on Nicotiana rustica as a source of nicotine for insect control. Journal of Economic Entomology, Vol. 18, No. 6, p. 807.

TABLE 5. THE YIELD AND NICOTINE CONTENT OF NICOTIANA RUSTICA TOBACCO AS INFLUENCED BY FERTILIZER TREATMENT AND SEASON

Acre treatment	Yield leaf and stalk	Nicotine content
	pounds	per cent
Acre treatment	Yield leaf and stalk	Nicotine content
	pounds	per cent
1924		
400 pounds sulphate of ammonia—400 pounds superphosphate—400 pounds kainit	3130	4.91
1200 pounds cottonseed meal—300 pounds superphosphate—200 pounds manure salt	2500	3.76
500 pounds tankage—400 pounds nitrate of soda—100 pounds superphosphate—400 pounds kainit	3075	5.05
600 pounds sulphate of ammonia—400 pounds superphosphate—400 pounds kainit	3000	4.47
600 pounds cottonseed meal—200 pounds tankage—200 pounds manure salt	2200	4.28
No fertilizer	1200	4.64
1925		
400 pounds sulphate of ammonia—400 pounds superphosphate—400 pounds kainit	3850	6.38
600 pounds sulphate of ammonia—400 pounds superphosphate—400 pounds kainit	2450	5.04
400 pounds nitrate of soda—400 pounds superphosphate—400 pounds kainit	3710	5.17
600 pounds nitrate of soda—400 pounds superphosphate—400 pounds kainit	3570	5.63

In the manufacture of tobacco dust, the entire plant is used. The following table shows the relative yields of leaf and stalk per acre:

Strain	Leaves pounds	Stalks pounds	Total pounds
34752. rustica, Russian	2,250	1750	4000
5. rustica, English	1,500	1000	2500
1. Cross between 34752 and 5	2,800	1500	4300

In addition to the fertilizer experiments, 74 strains of *rustica* tobacco obtained by the breeding work at Ephrata and Lock Haven, and by the agricultural explorers of the United States Department of Agriculture from various parts of the world, were tested as to yield and nicotine content during 1926 and 1927 at Lock Haven. A number of crosses were made from the most promising strains for further work.

Tobacco Curing Experiments

Proper curing of tobacco is, as a rule, somewhat neglected among Pennsylvania tobacco growers, though it is of considerable importance in successful tobacco production. The process of curing, during which the tobacco changes from a green, brittle, moisture-laden leaf to a brown and elastic one within 5 to 6 weeks, is complicated, perilous, and little understood. The curing sheds seldom are constructed so as to prevent losses which occur almost every year from pole-burn and other diseases attacking the tobacco while curing. Frequently haymows, corncribs, and wagon sheds, entirely lacking in ventilation facilities, are used for this purpose.

While it has been known for a long time that artificial heat in the curing shed will prevent poleburn, mildew, and the like, and reduce the length of the curing period, it had not been tried in Pennsylvania until the experiments at Ephrata proved its value. These experiments have been conducted since 1921, and include a comparison of salamanders heated with charcoal, a tobacco-curing furnace such as is used in the southern tobacco districts, and a pipeless furnace.

The salamanders, which are cylindrical in shape, are made of iron. They are open at the top and have a grate in the lower end. While fairly satisfactory, the charcoal fuel is rather costly, and any other free-burning fuel imparts a smoky taste to the tobacco. The tobacco hanging directly above the heaters is, as a rule, damaged by the heat, and since several salamanders are required for a large shed, constant attention is necessary.

The tobacco-curing furnace was placed in the wall of the experiment barn, and fired from the outside, with flues extending along the floor in the center to the opposite wall, returning to the smokepipe along the walls on both sides.

The heat generated from the central flue, however, was prone to set fire to the floor, so its use had to be abandoned. This type of furnace probably would be better adapted to tobacco sheds with dirt floors.

The pipeless furnace was placed in the stripping room, which is located in the cellar under the barn. The register extends up to the floor in the barn, which is partitioned into two sections, one of which is unheated and used for normal air-curing. Cold air flues, running from the corners of the heated section to the furnace, serve to draw down the cold, moisture-laden air. The heating is begun on the second day after the barn is filled with tobacco and continued until all danger of pole burn is past, which requires an average of about 16 days. The temperature of the heated section is kept from 15 to 20° Fahrenheit above that on the outside, except on warm days, which are of infrequent occurrence in Pennsylvania during the curing season. All ventilators, but top and bottom, are closed during the heating period, except for an occasional "airing."

There has been no damage from pole burn in the heated section since the installation of the pipeless furnace five years ago, while every year there is more or less damage in the unheated section. On an average, the tobacco cures in 25 days in the heated section, while normal air curing takes from 50 to 60 days. The pipeless furnace, once installed, may be used to provide heat during the stripping operations. With the use of a cheap fuel it should pay its cost in a few years, if weather conditions are unfavorable for proper curing. While it may be used only when there is a cellar beneath the shed, the modern heaters, although smaller, should prove satisfactory in barns that lack cellars. However, it has been shown that any method by which artificial heat may be applied at a reasonable cost during critical times in the curing season will prevent or greatly reduce damage from pole burn.

Tobacco Sweating Experiments

All types of cigar-leaf tobacco are subjected to a fermentation or "sweating" process before being manufactured into cigars. This process removes the raw taste, develops flavor and aroma, changes the leaf from a greenish to a dark brown color and causes a loss of some of the nicotine present and from 10 to 15 per cent of the original weight. The loss and changes of the chemical constituents of the leaf are considerable, but the process has not been investigated as fully as its importance demands.

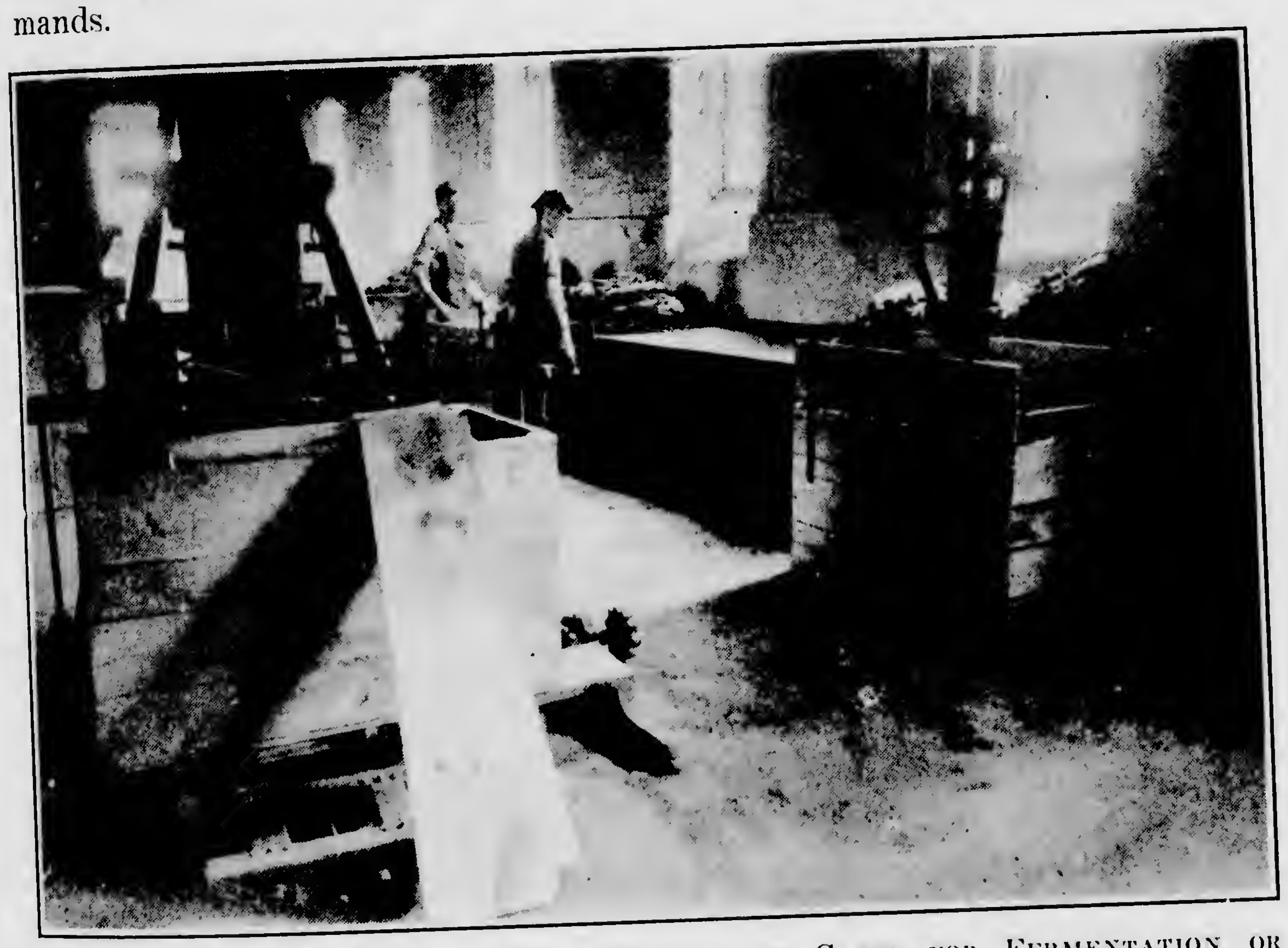

Fig. 7. Cigar-leaf Tobacco Being Packed in Cases for Fermentation or "Sweating"

Several methods of sweating tobacco are in use, but the one most generally practiced in Pennsylvania is that known as "natural sweat."

This is accomplished by packing from 300 to 400 pounds of tobacco, properly graded, into wooden boxes or cases which are fairly tight on the sides, but have one-half inch spaces between the end boards. (Fig. 7). The inside of the cases, except the ends, are covered with heavy paper. The "hands" of tobacco are packed with the tips meeting and overlapping in the center and with the butts toward the ends, leaving a 1½-inch space between the butts and the end boards of the case. When the tobacco has been pressed down and the lid nailed on, the cases are stored in unheated warehouses, being stacked from 5 to 20 high by means of traveling cranes or block and tackle. Here they are left undisturbed until the following fall, when the tobacco is sampled.

The packing operations are usually conducted during late winter and early spring, and the tobacco frequently is not in proper condition for the sweating process, having been packed in bales by the grower and stored in the barns and stripping rooms under widely varying conditions of moisture. There is no doubt that development of black rot, must, and moulds is caused by excessive moisture in the tobacco, and it is very difficult to determine the necessary amount of moisture needed for a good sweat.

During 1925 and 1926, experiments were conducted at Ephrata to secure data on the time required to start sweating after the tobacco is packed in cases; and whether the heating of the sweating room to 90° Fahrenheit or over would hasten the sweating process, thus tending to reduce or eliminate the danger of black rot and kindred diseases, which cause an annual loss amounting to many thousands of dollars. The data obtained indicate the entire practicability of these methods, experimentally, but the work should be repeated on a much larger scale than is possible with the experimental crops before reliable conclusions may be drawn.

The 1924 and 1925, experimental tobacco crops were used for this experiment. The bales were opened and the tobacco packed in the cases between, February 18 and February 20. Lacking a heavy press, it was impossible to compress the tobacco in the cases sufficiently to insure proper sweating. Before packing, 12 leaves were picked from the lots assigned to each case. Each leaf was weighed for a determination of the average moisture content of each case. The work was conducted in different compartments of the barn, as indicated below, in order to find out the effects of different temperatures on this process:

A.—The part of the stripping cellar partitioned off next to the furnace, with five cases placed on a platform near the ceiling.

B.—Same, with four cases on the floor.

C.—Another part of the stripping room, heated slightly by the furnace.

D.—The floor above the stripping room, without any heat.

The heating was begun February 21 and continued until April 1. For the first few days, or until the tobacco began to sweat, the heat was maintained night and day; later the furnace was checked at night. The tobacco in A began to sweat four days after the heat was applied. In B, it took 10 days; in C, 10 days, and in D, 15 days. With proper facilities it should be possible to heat the tobacco so that it would start to sweat in a day or two.

It was noted that the moisture content, size, texture, and body of the tobacco had considerable influence on the sweating process. Thus, tobacco with a moisture content of from 24 to 26 per cent and having a fairly heavy to heavy body (that is, rather thick in texture) sweated better than tobacco having the same moisture content, but a flimsy or rather thin body. When the moisture content was less than 23 per cent the tobacco did not sweat; when it was higher than 27 per cent, damage from black rot resulted. The following table shows the results of the experiment:

Table 6. Tobacco Sweating Experiments

Lot A. The room temperature ranged from 70 to 102° Fahr., with an average of 80° for the 20 days of heating.

Case No.	Size inches	Weight pounds	Moisture per cent	Condition of tobacco after sweating:
1	26	292	26.65	Sound. Well sweated. Thin to heavy body.
2	28	353	27.98	Slight black rot. Well sweated. Thin.
3	28	318	24.62	Sound. Well sweated. Medium heavy body.
4	20	224	23.88	Sound. Slight sweat. Flimsy.
5	22	140	22.85	Sound. No sweat. Flimsy body.
Lot B. The room temperature ranged from 55 to 80°, with an average of 65°.				
7	28	304	27.22	Slight black rot. Slight sweat. Thin body.
8	24	248	26.76	Sound. Slight sweat. Medium heavy body.
9	24	196	25.65	Sound. Very slight sweat. Flimsy body.
10	26	167	25.89	Sound. Very slight sweat. Flimsy body.
Lot C. The room temperature ranged from 35 to 80°, with an average of 60°.				
11	26	306	27.63	Badly damaged by black rot. Heavy body.
12	28	268	26.88	Badly moulded. Thin body.
13	16	268	25.05	Sound. Slightly sweated. Heavy body.
Lot D. The room temperature ranged from 30 to 64°, with an average of 48°.				
14	22	217	25.97	Sound. No sweat. Very heavy body.
15	24	264	26.49	Sound. No sweat. Heavy body.

Chemical Investigations Relative to the Burning Qualities

The burn, flavor and aroma of cigar-leaf tobacco constitute its three most desirable qualities. Of these, burn is probably the most important. For this reason, considerable emphasis was placed on the study of some of the factors which were thought to influence this particular quality directly or indirectly, Following the completion of the curing process for the years 1925, 1926, and 1927, samples of leaves were taken from a representative number of plants, which had been previously selected in the field. These samples were dried under the temperature prevailing in the laboratory, the stems removed, and the web finely ground. The samples were placed in air-tight containers and reserved for chemical analysis.

A number of analyses were run on both the 1925 and 1926 crops, the results of which are given elsewhere.[4] Data on the quantity of the most important mineral constituents in these crops are given in Table 7, which also contains the data relative to the potash content of the 1927 crop.

TABLE 7. THE PARTIAL INORGANIC COMPOSITION OF THE WEB OF CURED PENNSYLVANIA CIGAR-FILLER TOBACCO, HIBSHMAN STRAIN, AS MODIFIED BY THE SEASON AND FERTILIZER TREATMENT

Plot No.	Percentage—Composition										
	Crude ash		K_2O			CaO		MgO		Cl	
	1925	1926	1925	1926	1927	1925	1926	1925	1926	1925	1926
A-1	23.99	23.79	1.93	2.55	4.63	7.30	6.44	1.24	0.75	0.19	0.24
A-2	24.08	18.03	1.89	2.13	3.83	7.88	6.39	1.25	0.94	1.12	0.94
A-3	23.10	22.80	1.96	2.33	4.22	7.64	6.79	1.15	1.06	0.95	0.95
A-4	24.15	23.34	1.95	2.69	4.89	7.93	6.56	1.21	0.82	2.29	1.95
A-5	22.78	21.08	1.65	2.38	4.15	7.58	6.29	1.32	0.86	0.17	0.17
B-2	24.45	22.92	1.84	2.04	4.15	7.08	6.83	1.16	1.09	1.26	1.07
B-3	23.34	22.54	1.93	3.37	4.40	7.30	6.75	1.08	0.88	0.22	0.19
B-4	23.32	22.52	1.97	2.13	3.80	7.83	6.79	1.31	0.95	0.19	0.21
B-5	23.59	20.70	2.03	2.19	4.04	7.17	6.78	1.14	1.40	0.25	0.21

Table 7 shows that the potash content of the leaves was rather low while the lime content was relatively high. Analysis of the soil had indicated a lime content exceeding two per cent. The presence in the soil of soluble lime compounds, which should prevail under these conditions, leads to the unavailability of soil potash, so far as tobacco and similar plants are concerned. Table 7 likewise shows that the potash content of the 1926 samples was greater than that of the 1925 crop, while the lime content was somewhat the reverse. Weather observations led to this difference being attributed to the leaching of active lime compounds from the soil, coupled with the increased root development that should result from the moister, conditions that prevailed during the greater part of the 1926 season.

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[4] Haley, D. E., Nasset, E. S., and Olson, Otto. 1928. A study of certain constituents of the leaf and their relation to the burning qualities of tobacco. Plant Physiology, Vol. 3. No. 2, p. 185.

Probably no other single factor contributes more toward desirable burning qualities of tobacco than the potash content. A great deal depends, however, on the form in which the potash is present within the plant. For this reason, these samples were analyzed for sulphur, on the assumption that large quantities of sulphur may indicate the possibility of part of the potash being combined with sulphur, forming the sulfate. The analyses showed, however, that the samples of the 1925 crop contained a minimum of 1.14 and a maximum of 1.61 per cent of sulphur trioxide, and that additions of sulfate of potash did not materially increase the percentage of sulphur over those receiving no sulfate additions. The chlorine content, however, was found to vary according to the quantity of chlorine—containing materials used.

Observations have indicated that the favorable influence of potash on the burning qualities of tobacco is obtained when measurable quantities of this element are in combination with ether-soluble organic acids, and especially those containing hydroxy groups, as citric and malic acids. On the other hand, free acids are not so desirable, since the actual acidity of the plants may exceed the optimum concentration for the maximum activity of the oxidase enzyme, the activity of which is so important during the curing and fermentation of the tobacco, and for other reasons. Therefore, some attention was given to the study of the total ether-soluble organic acids present, to their possible state of combination, and to the H-ion concentration of the samples.

The results are given in Table 8, the alkalinity being expressed as the number of cc. of NH_2SO_4 required for the neutralization of the ash of 100 grams of the sample used:

TABLE 8. ALKALINITY OF THE ASH, THE H-ION CONCENTRATION AND THE ETHER-SOLUBLE ORGANIC ACID CONTENT OF THE WEB OF CURED PENNSYLVANIA CIGAR-FILLER TOBACCO, HIBSHMAN STRAIN, AS MODIFIED BY SEASON AND FERTILIZER TREATMENT

Plot No.	Alkalinity of ash						Ether-sol. org. acids		H-ion concentration	
	Soluble		Insoluble		Total					
	1925	1926	1925	1926	1925	1926	1925	1926	1925	1926
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	pH	pH
A-1	22	34	332	278	354	312	347	290	5.78	6.08
A-2	11	36	343	293	354	329	333	305	5.96	5.95
A-3	13	37	232	326	336	363	331	293	6.01	6.20
A-4	8	17	315	333	323	350	314	274	5.97	6.10
A-5	13	51	343	323	356	374	351	290	5.76	6.16
B-2	14	25	317	296	331	321	313	286	5.38	6.37
B-3	35	39	309	306	344	345	345	283	5.34	6.08
B-4	19	40	353	320	372	360	362	293	5.74	6.78
B-5	19	32	321	320	340	355	333	300	5.61	6.27

Table 8 shows that where chlorine was present in appreciable quantities, the alkalinity of the soluble ash was quite low, which indicates, in a measure, inorganic rather than organic forms of the potash present. The results also show that the greater the alkalinity of the soluble ash, the less the actual acidity.

After the 1925 and 1926 samples had undergone fermentation, a measure of the burning quality was obtained by means of the "strip test," the results of which are given in Table 8.

TABLE 9. STRIP TESTS OF THE 1925 AND 1926 CROPS AFTER FERMENTATION

Plot No.	Strip tests*	
	1925	1926
A-1	Poor	Poor
A-2	Poor	Poor
A-3	Poor	Poor
A-4	Poor	Poor
A-5	Good	Good to Fine
B-2	Poor	Poor
B-3	Fair to Good	Good to Fine
B-4	Good	Good to Fine
B-5	Fair	Fair to Good

* Definitions of terms in strip tests:
Very poor=tobacco does not hold fire. Coals badly.
Poor=tobacco holds fire but a few seconds. Coals badly.
Fair=tobacco holds fire over five seconds. Coals moderately.
Good=tobacco holds fire evenly over ten seconds. Coals slightly.
Fine=tobacco holds fire evenly until consumed. Coals very slightly.
Excellent=tobacco burns evenly until consumed without coaling and with light colored ash.

The results given in Table 9 show that the burn of the 1926 crop was, on the whole, uniformly better than that of the 1925 crop. Where chlorine was present, however, the burn was consistently poor.

TABLE 10. FERTILIZER TREATMENTS OF OUTLYING EXPERIMENTAL PLOTS

Plot No.	Manure	Cottonseed meal	Nitrate of soda	Carbonate of potash	Sulphate of potash	Bone meal	Precipitated bone phosphate	Acid phosphate
	Tons	lbs.						
A-1	2	610	130	155	------	------	140	--------
B-1	15							
A-2	--------	610	130	155	------	------	140	--------
B-2	10	--------	152	--------	26	------	--------	45
A-3	10	610	130	155	------	------	140	--------
B-3	10	300	--------	--------	350	350	--------	--------
A-4	--------	610	130	155	------	------	140	--------
B-4	--------	--------	100	--------	200	------	--------	160
A-5	10	610	130	155	------	------	140	--------
B-5	10	310	--------	--------	120	120	--------	--------
A-6	--------	610	130	155	------	------	140	--------
B-6	10	300	--------	--------	100	------	--------	100
A-7	--------	610	130	155	------	------	140	--------
B-7	20	--------	--------	--------				
A-8	--------	610	130	155	------	------	140	--------
B-8	20							

As a result of these investigations at Ephrata, it was decided to conduct somewhat similar experiments in different parts of Lancaster county. This work was made possible by the cooperation of eight different growers and a cigar company. An acre of ground, on a representative farm of the locality was selected in every case. On one portion, designated by A, an application of carbonate of potash, precipitated bone phosphate, nitrate of soda, and cottonseed meal was made. In some cases manure was applied also. The remaining portion of each plot, designated as B, was fertilized according to the judgment of the individual grower. The plots were numbered from 1 to 8, inclusive. The treatment of both portions of every plot is given in Table 10.

At the end of the curing period, samples were taken for analysis. Determinations were made for crude ash, chlorine, lime, magnesia, potash, and alkalinity of the soluble and insoluble ash. These analyses are given in Table 11.

TABLE 11. CHEMICAL STUDIES OF CURED PENNSYLVANIA CIGAR-FILLER TOBACCO, HIBSHMAN STRAIN, AS MODIFIED BY FERTILIZER TREATMENT AND LOCALITY

Plot No.	Crude Ash	Chlorine	CaO	MgO	K_2O	Alkalinity of the Ash		
						Sol.	Insol.	Total
A-1	20.83	None	6.55	1.12	5.04	33	321	354
B-1	20.71	0.74	5.64	1.09	4.63	38	310	348
A-2		Trace	5.44	1.70	4.27	31	319	350
B-2	19.71	0.65	5.43	1.37	4.19	37	290	327
A-3	20.69	Trace	5.52	1.11	5.13	50	217	257
B-3	20.05	Trace	5.19	1.54	5.03	29	291	320
A-4	24.77	None	5.68	0.96	4.91	73	215	288
B-4	20.77	1.21	5.49	0.60	4.79	48	264	312
A-5	20.53	None	5.71	0.78	4.72	63	236	299
B-5	21.45	Trace	5.62	0.83	5.24	65	275	340
A-6	20.16	None	5.91	0.98	4.32	36	286	322
B-6	20.88	Trace	5.45	1.10	5.40	48	287	335
A-7	20.27	None	6.67	0.92	3.39	29	242	271
B-7	20.67	None	4.89	0.89	4.42	38	263	301
A-8	20.98	Trace	6.63	1.59	4.27	31	325	356
B-8	19.76	0.50	5.55	1.61	4.64	23	302	325

These results show, on the whole, that the lime content was uniformly lower and the potash content correspondingly higher than the samples of the two preceding years taken from the plots at Ephrata. They also show that the locality in which the plants were grown, probably coupled with their previous history, overshadow the influence of the fertilizer treatments employed.

Summary and Conclusions

The experiments herein described have to do, in the main, with the development of desirable strains of cigar-leaf tobacco; the relation of field treatment to the yield, chemical composition and quality of the product obtained; the relation of artificial heat to the curing and fermentation processes, and the development of high-nicotine strains of tobacco which may be used for insect control. Strains of cigar-leaf tobacco have been developed which have high resistance to root-rot and which produce a high yield under favorable environmental conditions. Promising results have been obtained in the development of both high and low nicotine strains. The use of artificial heat has resulted in the prevention of pole-burn and a shortening of the curing period. Artificial heat was used to shorten the period of fermentation and prevent the development of black-rot during this process.

The use of manure alone has not proved satisfactory as a field treatment. The yield and quality of the plants produced under these conditions have been consistently inferior to those produced by applications of manure supplemented with commercial fertilizers. On the other hand, high yields and good quality tobacco were obtained when commercial fertilizers were used without manure.

Chemical studies of the leaves show that their composition may be materially affected by locality, weather conditions, and fertilizer treatment; and that, in turn, the chemical composition affects their burning quality.

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ON THE RELATION OF POTASSIUM TO IRON IN THE COMBUSTION OF CIGAR-LEAF TOBACCO[1]

IT has long been recognized that potassium may act as a catalyst in the combustion of cigar-leaf tobacco, and that its efficiency in this respect is largely determined by the form in which it is found in the leaf. These points have never been questioned seriously, but opinions have differed concerning the exact mechanism involved in this form of catalysis.

In our studies on the burning qualities of Pennsylvania cigar-leaf tobacco, we have substantiated the claims of others, namely, that ready combustion is usually, but not always, associated with potassium in organic rather than in inorganic forms of combination. Moreover, we found that if the ash of a cigar was rubbed into the surface of an ash-free cellulose filter-paper, the latter could be made to burn with a glow, instead of a flame, and that the ash from a good burning cigar was more efficient in this respect than the ash from a cigar of inferior burning qualities. This suggested a modification of Garner's method[2] for a study of the effect of individual ash constituents on the combustion of organic materials, apart from those of the cigar-leaf, inasmuch as both insoluble and soluble ash constituents could be used advantageously.

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[1] Published by permission of the director of the Pennsylvania Agricultural Experiment Station as Technical Paper No. 474.

This investigation was conducted in cooperation with Dr. W. W. Garner, of the U. S. Department of Agriculture, Bureau of Plant Industry, Office of Tobacco and Plant Nutrition, and Professor F. D. Gardner, of the department of agronomy of the Pennsylvania State College.

[2] U. S. D. A. Bur. Plant Ind. Bul. 105.

As a result of these experiments it was found that a number of ash constituents and many of the organic compounds of the leaf imparted glowing qualities to the filter-paper. Of these, however, potassium carbonate appeared to be more efficient than any of the compounds studied, although its use usually resulted in the production of a dark ash. It is our opinion that the catalytic action of potassium carbonate on the burning of filter-paper is due to the fact that the potassium ion forms auto-oxidizable compounds with cellulose derivatives, and that the carbonate itself aids in the dispersion of carbon particles, thus increasing the total surface for combustion. That moisture must play a part in the dispersion of carbon was indicated by the fact that a dry mixture of carbon and potassium carbonate did not burn more readily than carbon when used alone. We conclude, therefore, that what holds true for pure cellulose holds true also for leaf tissue. In the latter, however, a greater dispersion of other materials present may likewise take place, materials which may have a catalytic action as well. Among these may be mentioned iron oxides. The presence of a mere trace of these compounds aids in the combustion of dry carbon.

Potassium carbonate, mixed with iron oxides which were obtained by the combustion of organic iron salts, when applied to filter-paper, was found to have a more beneficial effect on the glowing capacity and thoroughness of combustion than when either material was used alone. This observation led us to anticipate a rather close relationship from the standpoint of combustion between potassium and iron as they occur within the leaf. Tests on fermented samples of tobacco failed to show the presence of either the ferric or ferrous iron. A test showing the presence of complex ions of iron, on the other hand, was readily obtained. We have reason to assume, therefore, that the iron must be present in combination with salts of hydroxy acids or related compounds, which normally occur in the leaf. Experiments on the effect of these complex salts on the combustion of filter-paper showed that potassium-iron-citrate, for example, was far more efficient as a catalytic material than ammonium-iron-citrate.

The tests on filter-paper were followed by tests on pure sucrose, used alone or with various other materials, and heated to high temperatures in porcelain dishes. It was found that when sugar, alone, was used, a large increase in volume was obtained, followed by a less rapid loss of water, and fair combustion was apparent until all the water was eliminated. On the other hand, when equal parts of potassium-iron-citrate were used, a less increase in volume was obtained, followed by a slow loss of water. In this instance, however, ready combustion was effected. If the flame was removed before combustion was complete, the mass was found to glow for a long period of time. On complete combustion, a coherent ash remained, containing iron oxides, uniformly distributed and in a very finely divided condition. A somewhat similar result could be obtained by using the ash of a good burning cigar, but not with ammonium-iron-citrate or similar compounds.

The iron remaining behind was found to possess magnetic properties, even when the temperature used exceeded that of a burning cigar. Tests on cigar ash yielded magnetic iron, but only where combustion was incomplete, no test being obtained where carbon was absent. It was thought that the force of attraction between the oxides and certain of the ash constituents was great enough to overcome the magnetic force, because magnetic iron added to an ash failed to respond to a magnetic field. On the other hand, where the iron was not well distributed, enough of the iron oxides became localized in quantities sufficient to overcome the attractive force of the ash particles.

It is suggested, therefore, that good combustion of cigar-leaf tobacco is associated with potassium compounds which give rise to potassium carbonate during combustion, and that the presence of such compounds may serve to intensify the catalytic effects of iron on this process.

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IS OSSIFICATION INFLUENCED SOLELY BY ULTRA-VIOLET LIGHT?

Owing to the increasing interest in the physiological properties of ultra-violet light in the prevention and cure of rickets, legweakness in poultry and nutritional paralysis in swine, the following data, obtained in 1926, may be of interest to workers in the field of light therapy.

At that time we were conducting routine determinations of the ossifying potency of different sources of light, by means of the bone-ash method.[1] The question arose relative to the effect of diffused laboratory light and ordinary electric illumination on the ossification process. This question was raised because of rather constant variations in the bone ash of rats which were reared in different parts of the laboratory. Rats whose cages were continuously shaded or darkened invariably manifested a lower rate of calcification as compared with their own litter mates on the same ration, but whose cages were less shaded during the ordinary working hours.

The animal laboratory is situated in a half-story attic on the third floor, and the building is almost entirely surrounded by tall trees. As a result, the windows, which are small, are partly shaded. Practically no direct sunlight can reach the windows and little of this can penetrate into the laboratory, and the windows are usually closed during the cooler months. Consequently, it is necessary to work by electric light most of the year. These lights consisted of 75-watt Mazda bulbs hung at a distance of about twelve feet above the floor. The cages, which consisted of wood frame and wire mesh, were of the apartment type, consequently, most of the animals were shaded most of the time.

To determine, if possible, the effect of laboratory light on the calcification rate, litter mates twenty-one days of age and weighing approximately forty grams were divided into four experimental groups, all of which received the Wisconsin rachitogenic ration.[2] Group I was placed in a cylindrical wire-mesh cage covered with loosely woven black muslin cloth in which an opening was made through which was introduced a 100-watt Mazda bulb which was allowed to burn eight hours per day. Group II was placed in a similar cage which was covered completely with the black muslin cloth. Group III was placed in a similar cage without cover of any kind and exposed to the ordinary laboratory light. Group IV was treated the same as Group II except that the covering material consisted of cardboard arranged in such a manner that light was excluded completely but with facilities for air circulation. The following results were obtained when the femur bones were dried, extracted and ashed at the end of a twenty-one-day feeding period, the percentage of ash being calculated on the dry, fat-free basis.

Group	Treatment	Bone ash per cent.
I	Mazda bulb 8 hours per day	31.82
II	Dark cage—muslin cloth	22.44
III	Laboratory light	25.33
IV	Dark cage—cardboard cover	16.65

An additional experiment was conducted in which the feeding period was lengthened to thirty-five days, the results of which were as follows:

Group	Treatment	Bone ash per cent.
V	Mazda bulb 24 hours per day	50.59
VI	Dark cage	18.46
VII	Laboratory light	26.48
VIII	Two drops cod-liver oil daily	38.95

While we have had no facilities for determining the amount of ultra-violet light to which these animals were exposed, we have every reason to believe that it must have been negligible, since most authorities are agreed that the production of ultra-violet light by the ordinary filament is small and that this is largely if not completely absorbed by the glass bulb. While the possibility is not excluded that the variations in bone deposition are due to ultra-violet light, we can not escape the conclusion that the longer wave-lengths also possess ossifying power.

These observations are in accord with our observations (R.A.D.) on poultry, in which we have noted definite superiority of birds reared on a legweakness-producing diet and exposed to sunshine, as compared with birds subsisting on the same ration but irradiated with ultra-violet light from the quartz mercury vapor lamp.

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[1] R. A. Dutcher, M. Creighton and H. Rothrock, *J. Biol. Chem.*, 1925, 66: 401.

[2] H. Steenbock and A. Black, *J. Biol. Chem.*, 1925, 64: 263.

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VITAMINS IN HUMAN AND ANIMAL NUTRITION.*

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One of the first researches, which led, eventually, to the vitamin hypothesis, was that of Lunin, who published a paper in 1881 stating that he had fed purified rations (to experimental animals) which were incapable of producing growth. The addition of a small amount of milk, however, caused the animals to resume their normal rate of growth. This paper was published in a journal of limited circulation, with the result that Lunin's observation was not brought to the attention of scientists for a number of years.

Probably the first piece of work to really stimulate interest in this field was that of Eijkmann (1897), a Dutch physician, who produced experimental Avian beri-beri by feeding polished rice to poultry. Eijkmann was able to show that the polishings or branny portion possessed curative properties and, later, he succeeded in curing and preventing this deficiency disease by feeding or injecting an extract of rice polishings. He concluded that white rice contained a toxin or poison that was the prime etiologic factor in beri-beri, and he postulated that the curative properties of the polishings were due to the presence of a substance which acted as an antidote. In spite of this incorrect conclusion, his work attracted considerable attention and stimulated further work by other investigators.

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Another important series of observations that paved the way for vitamin research were those of Hopkins of England. Dr. Hopkins showed, as early as 1906, that as little as 2 cc. of cow's milk caused rats to grow on purified diets. He pointed out that this stimulation could not be due to fats, proteins, carbohydrates or mineral salts. He postulated that milk must contain some hitherto unknown "accessory food factor." He is given the credit, therefore, for suggesting the existence of these food factors that we now call vitamins.

Between the years 1910 and 1912 Dr. Casimir Funk repeated and substantiated the work of Eijkmann. Being a chemist, however, he pursued the problem further, with the result that he was able to isolate from rice polishings a crystalline chemical substance which contained carbon, hydrogen, oxygen and nitrogen. This substance was curative in avian beri-beri and possessed basic properties. As a result, Funk suggested the name "vitamine" for this food factor. Some years later the "e" was dropped from the word "vitamine" in order that this class of substances should not be confused with the plant alkaloids such as strychnin, morphin, etc.

The first systematic work in vitamin research in this country developed in the laboratories of Osborne and Mendel at Yale and in the laboratories of Babcock, Hart and McCollum at Wisconsin. It was not long before these laboratories obtained proof for the existence of a fat-soluble food factor in cod liver oil, butter fat and egg yolk. The absence of this factor from the diet caused loss in weight, accompanied by the development of xerophthalmia. At about the same time McCollum and Kennedy showed that the antineuritic factor of Funk possessed growth promoting properties when fed to rats. McCollum and his co-workers were unable to find that the fat-soluble factor contained nitrogen and for that reason refused to adopt the term "vitamine." They suggested, therefore, that the fat-soluble factor be known as "Fat-soluble A" and that the water-soluble factor in rice polishings and in yeast be called "Water-soluble B."

Lack of time prevents further discussion of the discovery of the various vitamins. I wish to say, however, that the term vitamin appealed to popular and scientific fancy, with the result that it has become official and universal in usage.

Then came the discovery of vitamin C, the antiscorbutic factor, which was found in citrus fruits, sprouting grains, green leafy plants and in many vegetables and fruits.

For a time the work of Mellanby and others seemed to indicate that vitamin A also possessed antiratchitic properties. Mellanby also noted that dogs developed rickets in confinement much more rapidly than when they were allowed to run out of doors. At that time he was inclined to believe that exercise was curative or at least preventive in rickets. Later work on heliotherapy, however, has provided an explanation for his results.

McCollum and co-workers were able to destroy vitamin A in cod liver oil without injuring the antiratchitic potency of the oil appreciably. This work led to the introduction of the term "vitamin D" to designate the antirachitic factor.

A few years later Dr. Herbert Evans of California introduced proof for the existence of an antisterility factor which he first called "vitamin X," but later the term "Vitamin E" was adopted. This factor, according to Evans, brings about fertility when rats have been made temporarily sterile on certain types of rations. He finds this factor quite universally distributed, but finds it in high concentration in cereal oils, particularly wheat germ oil. This factor is capable of withstanding saponification and distillation.

Recently the California workers have suggested a new factor called "vitamin F," but since some investigators are questioning its existence I shall not take time to discuss it.

Within the past few years evidence has accumulated to prove that the old vitamin B is really a "complex," consisting of at least two, and possibly more factors. As a result vitamin workers have given scientific recognition to two fractions. The first one, which cures beri-beri and which is heat labile is now called "vitamin B" by American workers and "vitamin B_1" by British workers. The second fraction of the old vitamin B complex, which promotes appetite, cures skin lesions (pellagra) and which is thermo-stable, is now called "vitamin G" by American workers and "vitamin B_2" by British workers.

Vitamin A is found in the unsaponifiable portion of those fats which contain it. It is fairly stable to heat in the absence of oxygen, but in the presence of oxygen it is destroyed. Even in the absence of heat it is susceptible to slow oxidation. In a general way the vitamin A content of foods is related to pigmentation—i. e., those foods which are rich in the carotinoid pigment are usually rich in vitamin A. There are exceptions to this rule, however.

Vitamin B, the antineuritic factor, is soluble in water and water-alcohol solutions. It can be precipitated, dialyzed, and adsorbed on the surface of colloids. It is quite easily destroyed by heat as compared with vitamin G.

Vitamin C, the antiscorbutic factor, is also water-soluble, dialyzable and is stable in acid solutions but destroyed by alkalies. It is also very susceptible to oxidation, but may be considered fairly stable to heat if oxygen is absent.

Vitamin D, the antirachitic factor, is quite stable to heat, is soluble in the fat solvents, is stable to saponification reactions and is associated with the sterol fraction of those fats containing it. Recent work indicates that the alcohol "ergosterol" is identical with or is the parent substance of vitamin D.

Vitamin E, the antisterility factor, as I have already indicated, is fat-soluble, heat stable and is found in the unsaponifiable fraction of wheat germ oil.

Vitamin G, the appetite promoting, pellagra preventive factor, is soluble in water and water-alcohol solutions. It is not absorbed at the same hydrogen-ion concentration as the antineuritic factor, and from its deportment it is thought that it is a nitrogenous compound also. Much of the old work on vitamin B must now be repeated, before we can interpret all data correctly.

MEDICAL AND NUTRITIONAL ASPECTS.

May I explain that the literature is full of ideas and opinions, many of which are unsupported by experimental evidence, relative to the physiologic function of vitamins? I shall attempt to mention but a few.

Functions of Vitamin A.—Some writers believe that vitamin A promotes the function of tissues and that blood platelets fluctuate with the supply of this food factor. It is certain that appetite, growth and well-being are dependent on this vitamin. A deficiency of vitamin A causes loss of appetite, loss in body weight and susceptibility to infection, particularly of the eyes, ears, sinuses, air passages and lungs. In the advanced stages of vitamin A deficiency we find xerosis, corneal ulcers, colds, influenza, pneumonia, otitis media and, occasionally, infections at the base of the tongue. Renal calculi have also been noted in many cases. Diets containing adequate amounts of cod liver oil, butter fat, cream, carrots, liver, etc., prevent the pathologic conditions just described.

Functions of the Vitamin B Complex.—Owing to our lack of knowledge regarding the new factors B (or B_1) and G (or B_2), I shall discuss them together. When the complex is omitted from the diet appetite fails and losses in body weight and emaciation ensue. Death may occur with or without the development of polyneuritic symptoms. Usually, the animals are constipated and are characterized by marked loss in vigor.

In all stages of vitamin deficiency there is a characteristic loss of sexual interest and, consequently, failure to breed. My own work and that of other investigators shows that there is a tendency for all vital organs of the body to atrophy in vitamin B (complex) deficiency with the exception of the suprarenal glands, which show a distinct tendency to hypertrophy.

There seems to be some relationship between nuclear nutrition and vitamin B (complex) supply. McCarrison has been so positive of this that he has suggested that we adopt the term "nucleopast" to emphasize that these factors stimulate nuclear metabolism. I have already noted that the absence of vitamin B (B_1) induces polyneuritis and that vitamin G (B_2) has to do with appetite stimulation and the prevention of pellagra. Our own researches have indicated that this complex may function, directly or indirectly, as a stimulator of the oxidative processes, but this theory has been questioned by some investigators.

The vitamin B complex may be obtained from yeast, whole grains, vegetables, egg yolk, fruits, leafy vegetables and milk.

Functions of Vitamin C.—This is the antiscorbutic factor and, while necessary for many animals, does not seem to be necessary for rats or poultry. Guinea pigs are very susceptible to diets deficient in this factor. In the guinea pig we find that the functions of vitamin C and the pathology of scurvy are similar to if not identical with those observed in human scurvy. When the diet is deficient in this factor we note first loss in appetite and body weight, followed by weakness, rapid respiration, abnormal posture, swollen and tender joints, hemorrhagic gums, loss of teeth and paralysis. Postmortem examination reveals muscular hemorrhages, hypertrophy of adrenals and friable bones.

Orange juice, lemon juice, tomato juice, sprouted grains, leafy vegetables and fruits are good sources of vitamin C.

Functions of Vitamin D.—This is the antirachitic factor which plays a part in controlling calcium-phosphorus equilibrium and deposition. The outstanding symptom of vitamin D deficiency is the softness of bones and teeth. The ratio of calcium to phosphorus in the diet is a most important factor. Calcification is enhanced under all conditions, however, by vitamin D.

Recent work by Windaus, Rosenheim and Webster, Hess, Steenbock and others has led to the conviction that the alcohol ergosterol is the parent substance or precursor of vitamin D. The treatment of inactive ergosterol with ultraviolet light produces an antirachitic compound which is at least 100,000 times more potent than cod liver oil. The potency of antirachitic agents may be studied by four methods—i. e., (1) X-ray, (2) line test of bones, (3) blood phosphorus, or (4) mineral deposition, as measured by chemical analysis of the bone.

I shall not discuss the pathology of nutritional sterility and vitamin E function, owing to the lack of time, except to say that Evans and his co-workers describe several causes of

sterility, such as (1) failure of fertilization, (2) fertilization takes place but implantation does not, (3) fertilization and implantation occur but the fetus dies and is resorbed.

At this point Professor Dutcher showed about 30 lantern slides descriptive of the various types of vitamin research in his own and in other laboratories. He described his own work showing how it was discovered that the vitamin content of milk is dependent on the vitamin content of the diet. He described recent experiments in which he has found that rats cannot utilize vitamin A when butter fat is fed in juxtaposition to mineral oil or liquid petrolatum. He explained that this was not due to increased intestinal motility accompanied by rapid bowel evacuation. Neither did it appear to be a matter of intestinal permeability. He expressed the belief that it is most likely due to solubility of Vitamin A in the mineral oil, which is not assimilated.

Professor Dutcher also described the cooperative research of Bechdel, Dutcher and Knutsen in which they proved that the dairy cow could live, grow and reproduce on diets deficient in the Vitamin B complex. Subsequent research by means of a gastric (rumen) fistula proved that the cow is capable of developing bacteria in the rumen which synthesize sufficient vitamin B complex for her needs. An organism was isolated which caused rats to grow on diets deficient in the B complex. This organism, which had not been described, has been named "Bacterium Vitarumen".

In closing, he discussed ultra violet light and food irradiation and how we should make practical applications of our knowledge. He summarized his discussion by stating that while deficiency diseases are existent and important, that probably our main problem was not to worry about the development of deficiency diseases but rather to try to feed sufficiently varied diets to prevent lowered resistance and susceptibility to other diseases. He stated that, in the great majority of cases, it was possible and preferable to obtain our vitamin supply from the dairy, the garden, the orchard and the poultry yard.

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BALANCED FERTILIZERS AND LIEBIG'S LAW OF THE MINIMUM

LIEBIG maintained that "by the deficiency or absence of *one* necessary constituent, all others being present, the soil is rendered barren for all those crops to the life of which *that one* constituent is indispensable."[1] This afterwards became known as the "law of the minimum," from which it must logically follow, as indeed Liebig himself announced,[2] that nutrient elements are absorbed in certain definite proportions ("chiffres proportionals") in such a manner that a limit having been reached by one, the absorption of the others is retarded (depressed) *ipso facto*.

Accordingly, if with time as the abscissa and the quantity of nutrient element absorbed during the growth of a plant as the ordinate, graphs are plotted indicating the course of absorption of the three principal fertilizer constituents, nitrogen, phosphorus and potassium, from a soil to which a "complete" fertilizer has been added, it will follow from Liebig's basic assumption that, with all other growth factors constant, the graph showing the course of absorption of *any one* of these elements from a fertilizer containing only two of them would be displaced from its former position and will now fall below it.

The observed facts, however, were not always in harmony with Liebig's hypothesis. For example, from Liebig's assumption it should follow that the relationship between the supply of a particular plant nutrient and the dry matter produced would be one of simple proportion, and indeed this was Liebig's conception.[2] But this is not the case,[3, 4, 5] for the curve of dry matter produced plotted against nutrient supply is sigmoid in shape and not a straight line having the x and y coordinates equal to one another as would be expected if Liebig's deduction held true.

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[1] Justus Liebig, ''Chemistry in Its Applications to Agriculture and Physiology,'' third edition, 1843.

[2] Justus Liebig, ''Les lois naturelles de l'Agriculture,'' 2, second chapter, 1863.

Liebscher[6] suggested the substitution of his "law of the optimum" for the "law of the minimum." In effect, this is that the plant "is able to utilize to a greater extent that growth factor present in minimum amount the more the other factors already present approach toward their optimum values." But since water was the only growth variable tested by Leibscher, generalizations are hardly possible.

A few years later Mitscherlich[7] announced his "Gesetz der physiologischen Beziehungen," according to which any one individual growth factor can, independently, increase the growth of all others, provided it is not already present in optimum amounts. Mitscherlich's "law" has been variously interpreted. Some authorities have argued that it furnishes additional support for Liebig's "law of the minimum" and others consider that Liebig's and Mitscherlich's views are irreconcilable.

It has been argued[8] in favor of the retention of the "law of the minimum" that as shown by the experiments of Henrich,[9] Langer[10] and Lemmermann[8] certain applications of the "law" have been of value in explaining to the farmer certain basic principles of manuring.

No direct proof, however, against Liebig's "law of the minimum" has been forthcoming until Lagatu and Maume,[11] of l'Ecole Nationale d'Agriculture de Montpellier, in a series of brilliant investigations begun in 1919, showed that the course of absorption of nitrogen, phosphorus and potassium in the vine was irreconcilable with Liebig's "law." Owing, possibly, to the inaccessibility of some of the journals in which these investigations have been published, they have, unfortunately, passed unnoticed in this country.

The investigations have centered upon the analysis of the leaves of vines from experimental plots treated with "complete" fertilizer (N, P and K) and with combinations in which one of each of the elements is absent. Plots having no fertilizer applied serve as controls. Analyses with respect to N, P and K were made at regular intervals from May to October of each year. They have found the following striking results:

(1) The application of an "incomplete" fertilizer deficient in one of the elements N, P or K results in an *increased absorption by the plant of the other two.*

(2) This lack of balance in a fertilizer containing two only of the principal fertilizer constituents (N,

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[3] A. Hellreigel and H. Wilforth, *Ztschr. des Vereins f. d. Rübenzucker-Industrie*, 1888; *Arb. Deut. Landw. Gesell.*, 34: 18, 1898.

[4] H. Weissmann, *Ztschr. Pflanzenernähr. u. Düngung*, 2A: 1–79, 1923.

[5] E. J. Russell, ''Soil Conditions and Plant Growth,'' p. 36, fifth edition, 1927.

[6] G. Liebscher, *Jour. für Landw.*, 43: 49–216, 1895.

[7] Alfred Mitscherlich, *Landw. Jahrbücher*, 56: 71–92, 1921.

[8] O. Lemmermann, P. Hasse and W. Jensen, *Ztschr. Pflanzenernähr. u. Düngung*, B7: 49–98, 1928.

[9] R. Henrich, ''Dünger und Düngen,'' Preisschrift, p. 2, third edition, 1896.

[10] L. Langer, *Jour. für Landw.*, 49: 209–229, 1901.

[11] H. Lagatu, ''Rapport présenté au Congrès d'Agriculture régionale du Sud-Ouest,'' Toulouse, Mai, 1922, pp. 3–21; ''Communication au Congrès de l'Association française pour l'avancement des Sciences,'' Montpellier, *Juillet*, 1922, pp. 22–31; *Compt. Rend.*, 172: 129, 1921. H. Lagatu and L. Maume, *Compt. Rend.*, 179: 782, 1924; *ibid.*, 179: 932, 1924; *ibid.*, 180: 1179, 1925; ''Communication au Congrès des engrais azotés de synthèse à Montpellier,'' Juin 1, 1927, pp. 1–15; Extrait des *Compt. rend. l'acad. d'agric. France*, Avr. 6–Mai 11, 1927, pp. 1–24; *ibid.*, Juin 13, 1928, pp. 1–15; ''Communication faite le 22 Juin, 1928, à Bordeaux au Congrès intern. du vin et du pin maritime,'' pp. 1–12.

P, K) may be so great that the relative excess of these elements will "act as a brake" on the absorption of the one omitted to such an extent that *the plant will absorb from the soil containing this "incomplete" fertilizer less of the missing element than it would from the soil containing no fertilizer additions.*

(3) A lack of nutritional balance resulting from the addition of an unbalanced fertilizer results (except in the case of N deficiency) in decreased yields. This exceptional behavior of nitrogen is explained on the basis that this constituent is utilized more by the leaves than by the fruit (grapes). Development of the stems and leaves, however, will be retarded.

Both (1) and (2) above are contrary to Liebig's "law." Lagatu and Maume's results show that whatever effect the absence of an element from a fertilizer has on the yield, *it is not due to a depression of absorption of the other elements, but, on the contrary, to a nutritional lack of balance due to increased absorption of the remaining elements.*

It will be of interest to know if the remarkable results obtained by Lagatu and Maume in the case of the vine will be found to be true also for other plants. This point is now under examination for apple-trees grown under controlled conditions.[12]

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[12] W. Thomas and R. D. Anthony, *Proc. Am. Soc. Hort. Sci.*, pp. 81–87, 1926.

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HONEYWELL, H. E., R. ADAMS DUTCHER and J. O. ELY (STATE COLLEGE, PENNSYLVANIA). **Curative Technique in Vitamin A Studies as Influenced by Yeast from Various Sources.**

Four commercial yeast preparations, designated respectively as I, II, III and IV, were fed, in varying amounts, to rats in curative tests for vitamin A. Yeast I possessed the anti-xerophthalmic factor but there was no evidence of the presence of the growth promoting properties which are characteristic of vitamin A. Yeast II (made by the same company, two to three years later) possessed measurable amounts of the growth promoting (A) factor, but practically all of the experimental animals were afflicted with xerophthalmia.

Yeasts III and IV (manufactured by other companies) possessed no anti-xerophthalmic properties nor was there any evidence of the presence of the (vitamin A) growth factor. When two drops of butter fat were added at the end of the period, after the body stores of vitamin A were exhausted, the growth response seemed to be dependent on the kind and amount of vitamin A-free yeast used as the source of the vitamin B complex.

Subsequent studies, in which these yeasts were fed in varying amounts during *a*, the depletion period, and *b*, the curative period, have led to the following conclusions:

1. That yeast may contain vitamin A.
2. That some evidence was obtained to indicate the multiple nature of vitamin A but, owing to the exhaustion of the supply of this yeast and our inability to obtain additional samples, this phase of the work could not be continued.
3. That our inability, in the past, to obtain the proportion of xerophthalmia in our rat colony that has been reported by other workers has been explained.
4. That the quality and quantity of the vitamin B complex exerts a definite effect on growth response, even when a constant source of vitamin A (butter fat) is supplied.
5. That the quality and quantity of the vitamin B complex in the preventive and curative periods influence the growth response (when 2 drops of butter fat are added) in a manner which leads us to believe that the size of the animal, at the beginning of the curative period, is not the sole explanation.
6. That comparative quantitative determinations of Vitamin A in foods are not possible unless each new supply of yeast is tested and the technique standardized. We have obtained differences in curative response, on 2 drops of butter fat, which vary from 100 to 300 per cent, depending upon the kind and amount of yeast ingested; for example, 0.2 gram of yeast IV was superior to 0.6 gram of yeast I.

VITAMIN STUDIES XVII.
OSSIFYING POTENCY OF RAW AND EVAPORATED MILKS*

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Received for Publication—July 18, 1929

The investigations described in this paper are a continuation of our work on the vitamin content of raw and evaporated milk. Previous papers have dealt with the amount of vitamin A and of the B complex found in the milk of the Pennsylvania State College herd, and in the evaporated milk made therefrom. The present data represent an attempt to evaluate the ossifying potency of whole and treated milk.

In regard to the antirachitic potency of milk, published data exhibit conflicting results. Steenbock, Hart, Hoppert and Black (1), using their yellow corn ration (No. 2965), found that 12 cc. (daily) of cows' milk were the minimum requirement for the healing of rickets in rats. Luce (2) reported that, 2 to 5 cc. of milk from pasture-fed cows would prevent rickets in rats fed on the McCollum ration (No. 3143), but that 15 to 20 cc. of milk from cows kept in the dark were ineffective. Hess and Weinstock (3) stated that, in an experiment of the preventive type, 20 to 25 cc. of milk prevented rickets. Outhouse, Macy and Brekke (4), using the Osborne and Mendel rachitogenic ration, and a curative technique, found that "30 cc. of cows' milk fed daily for 7 days induced marked healing of rachitic lesions in rats." Differences in the environment and feeding of the cows as well as in rations, in animals, and in the experimental technique, make explanation of these conflicting results difficult.

In the present experiments the milk came from the college certified herd which is kept under conditions carefully standardized as to rations, exercise, and light. As described in our previous papers (5, 6), the evaporated milks were made from the same sample as the raw milk fed, and under conditions approximating those used in commercial practice. In this way the evaporated milks could be tested before and after sterilization. From June until January five kinds of milk, raw (RM), vacuum evaporated (VE), vacuum-evaporated-sterilized (VES), air-evaporated (AE), and air-evap-

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orated-sterilized (AES), were fed. Before feeding, the evaporated milks were diluted to the concentration of the raw milks in order to compare them with equal volumes of the raw certified milk from which they were made.

The animals were chosen carefully from the "Penn State strain" of pied rats reared in this laboratory on a diet the constituents of which have not been varied for several years (7). Each animal was placed in an individual cage and given the Steenbock yellow corn ration (No. 2965) (8) for 21 days, with the prescribed volume of milk in separate doses daily. Lighting conditions were uniform throughout, and no sunshine, even through glass, was allowed to fall on the cages. Equal numbers of male and female rats, 21 days of age and weighing between 36 and 42 grams, were evenly distributed throughout the experiment. At this age the amount of ash in the femurs of our experimental animals averaged about 42 per cent. At the end of the 21-day feeding period, the rats were killed with ether and examined carefully for beading of ribs, enlargement of joints, and softness of bones. The right humerus was preserved for the line test of McCollum and co-workers (9), and the femurs were removed for ashing according to our previous technique (7). The bones were dried at constant temperature in the electric oven for 24 hours, extracted with 95 per cent alcohol, and again with ether for the same length of time, dried, and ashed in a muffle furnace at about 600° C. On the basis of the dry extracted bone, the percentage of ash and the ratio of ash to organic matter (A/R) were calculated.

The data are summarized in Table I. The bone-ash recorded represents group averages and when distinct groups were fed the same quantity of milk at different seasons, the averages are given separately and, later, together. The raw milk was fed in 5, 10, 12, 15, and 20 cc. portions. The 5 and 10 cc. doses produced a bone-ash of 35 and 39 per cent, respectively, and both were below the original 42 per cent level of the bone-ash in the femurs at the time when the animals were placed on the experimental diet. The addition of 12 cc. of raw milk raised the bone-ash to 43.4 per cent, which is higher than the original level and yet not so high as the ash (53 per cent) produced in animals of the same age which have been fed on the stock ration for the same length of time. Since 5 and 10 cc. of raw milk (daily) did not increase the bone-ash and, since the ingestion of 20 cc. was very difficult for such small animals during the first part of the feeding period, 10, 12 and 15 cc. quantities were chosen as the levels at which the evaporated milks were to be fed.

The milks were fed from June, 1926, to January, 1927, and during July, 1927, the experiment was continued as before with the exception that equal amounts of milk-ash were fed to ascertain the contribution made by the minerals alone. The seasonal variations in ossification were not marked in animals receiving 10 cc. except in the group receiving raw milk, which may have been due to the feeding of small amounts of green feed to the college herd in June, the month in which one of the two determinations on raw milk was made. On the 12 cc. level seasonal variations were evident, in all the experimental groups in which two seasons were represented, and since in each case July was one of the months represented in the group, the ration given to the cows may account for the difference in the ossifying potency of the milk. This coincides with the experience of Luce (2) who found greater "antiricketic value" in milk from the same cow when she was pasture fed than when eating a dry ration.

The experimental milks were fed at the 10 cc. level and the percentages of bone-ash obtained were as follows: RM 38.3, VE 33.4, VES 27.8, AE 29.5, AES 25.5. When 12 cc. were fed, the percentages of bone-ash were RM 43.4, VE 39.3, VES 38.1, AE 35.0, AES 34.8. At the 15 cc. level, the percentages of bone ash produced were RM and VE 44.8, VES 44.1, AE 42.5, and AES 40.3. The differences between the milks are less marked at this level, possibly because, with this quantity, both the minerals and the calcifying potency of the milk are increased to the point where deposition is equal or superior to loss of bone salts.

According to Daniels and Loughlin (10), some of the differences between the evaporated and raw milks may be due to the mechanical removal of a portion of the mineral salts from the milk by the evaporation process. Evaporated milks made from the same original sample by the vacuum method and by the air-evaporation method, when fed in the same amounts produce a distinct and different lowering of the bone-ash. A difference in the method of evaporation might produce a difference in the mineral content and, therefore, a difference in the amount of ash in the bones of animals to which the two types of milk were fed, but when especial care was taken to prevent the loss of minerals by the use of glass containers, sterilization again produced a lowering in the bone-ash. Therefore, some of the variations in the bone-ash may have been due to loss of antirachitic potency as well as to direct loss of minerals in the evaporation process.

The bone-ash of the animals fed 20 cc. of raw milk averaged 48.8 per cent. This is nearly a normal value and agrees with the statement of Krauss (11), using 0.8 gm. of butter fat, that 23 cc. milk are required for normal bone formation in rats fed a rachitic ration. Our data on 20 cc. of raw milk and on evaporated milks are not in agreement with Daniels and Jordan (12), but, owing to lack of detailed data in their preliminary report and the apparent differences in technique, we are unable to explain the discrepancies at the present writing. Furthermore, the amount of antirachitic factor in our milks must have been much less than that observed by Daniels and Jordan, since they irradiated their milk samples with ultra violet light. It has been our experience that it is often necessary to feed a vitamin at the "threshold" level in order to note, with certainty, its susceptibility to heat treatment.

To determine the effect of the minerals alone on the rate of ossification, samples of raw, vacuum-evaporated, and vacuum-evaporated-sterilized milks were ashed. The ash was dissolved in dilute hydrochloric acid and made up with distilled water to the original volume of the milk from which the ash was prepared. One group of rats were fed 12 cc. aliquots, while their litter mates received the same volume of the original samples of milk. Unfortunately, at this time, air-evaporated milk samples were not available, since the evaporator had been removed from the dairy plant on the assumption that no more milk would be needed for this experiment.

The results of the feeding of the milk ash are shown in Table I. The bone of the rats which received ash from raw milk averaged 36.3 per cent, 9 per cent lower than their litter mates which were fed the same volume of raw milk. The separate groups of animals which received the two evaporated milks yielded approximately the same bone-ash, 33.8 and 33.7 per cent. The difference between the results given by the vacuum-evaporated milk and the same milk sterilized is practically negligible. The three groups which received milk ash produced a bone-ash which does not differ materially from that obtained with the air-evaporated milks, sterilized and unsterilized. These are the samples on which the greatest amount of injury was noted.

According to Chick and Roscoe (13), "The larger variations in bone composition seem to be confined to the water, fat, and mineral constituents. It seems probable that the value of the ratio, mineral ash: organic residue, might after all be the most trustworthy guide to the efficiency of the process of calcification." Therefore, the A/R ratios were calculated and are given in the table. Compared with the percentage of bone-ash they show the same general trend, *i.e.*, as the amount of bone-ash grows larger, the A/R ratio increases.

The line tests showed that the wide and irregular bands of cartilage had little or no deposition of calcium salts in the provisional zone of calcification at their base. As the cartilage bands became narrower and straighter, the line at the bases became heavier, showing that calcium salts had been

TABLE I

Milk	cc.	Month	No. of animals	Bone Ash per cent	A/R	Mean Ash	Mean A/R	Line test cartilage band
RM	5	June	8	35.2	.542	35.2	.542	
	10	June	7	40.0	.667	38.3	.622	Wide—fairly regular
		Dec.	8	36.9	.584			
	12	Dec.	12	41.7	.716	43.4	.775	Wider than normal bone—regular
		July	10	45.5	.846			
(*RM Ash*)	12	July	10	36.3	.572	36.3	.572	Wide—irregular
	15	Aug.	10	44.9	.817	44.8	.812	Narrower than 12 cc. RM—regular
		Oct.	4	44.6	.805			
	20	Aug.	8	48.8	.953	48.8	.953	Nearly like normal bone—regular
VE	10	Sept.	9	34.2	.521	33.4	.502	Wide—irregular
		Dec.	6	32.2	.475			
	12	Jan.	10	36.9	.586	39.3	.651	Wider than 12 cc. RM—regular
		July	10	41.7	.716			
(*VE Ash*)	12	July	10	33.8	.513	33.8	.513	Wide—irregular
	15	Oct.	9	44.8	.812	44.8	.812	Wider than 15 cc. RM—regular
VES	10	Sept.	10	27.7	.384	27.8	.388	Very wide—irregular
		Dec.	6	27.9	.395			
	12	Jan.	10	35.4	.548	38.1	.618	Wider than 12 cc. VE—irregular
		July	10	40.7	.688			
(*VES Ash*)	12	July	10	33.7	.511	33.7	.511	Very wide—irregular
	15	Oct.	10	44.1	.778	44.1	.778	Wider than 15 cc. VE—fairly regular
AE	10	Sept.	10	29.3	.414	29.5	.420	Very wide—irregular
		Dec.	6	29.8	.432			
	12	Jan.	10	35.0	.540	35.0	.540	Wider than 12 cc. RM—irregular
	15	Jan.	10	42.5	.743	42.5	.743	Wider than 15 cc. RM—regular
AES	10	Sept.	9	25.4	.340	25.5	.344	Very wide—irregular
		Dec.	5	25.8	.351			
	12	Jan.	10	34.8	.534	34.8	.534	Wider than 12 cc. AE—irregular
	15	Jan.	10	40.3	.673	40.3	.673	Wider than 15 cc. AE—fairly regular
Breeding ration						52.9	1.12	Narrow band—very regular

laid down. This band became narrower and more regular as the A/R ratio and the percentage of the bone-ash increased.

SUMMARY

Prophylactic experiments are described in which milk was added to a rachitic diet. Evaporated milks made by vacuum and aeration methods were fed at different levels in direct comparison with the raw milk from which they were made. A slight seasonal variation is shown in both the raw and evaporated milks.

The ossifying potency of the treated milks is less than that of the raw. Vacuum evaporation, aeration, and sterilization tended to decrease the ossifying potency of raw milk.

Ash, equivalent to 12 cc. of raw, vacuum-evaporated, and vacuum-evaporated-sterilized milk, was fed in comparison with the original samples. In all cases the milk-ash showed less ossifying potency than the milks from which they were obtained, indicating that a part of the calcifying properties of the various milk samples must have been due to the presence of vitamin D.

The data obtained indicate that the milks described are not rich in vitamin D. While commercial methods of evaporation and sterilization evidently affect the ossifying potency of milk to an appreciable degree, no attempt has been made to differentiate between mineral loss and vitamin D destruction.

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THE COMPOSITION OF NORMAL RAT BLOOD.*

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(Received for publication, November 27, 1929.)

A review of the literature on the composition of rat blood shows that very little work has been done in this field. In 1913 Folin and Morris (2) published data on the uric acid, non-protein nitrogen, and urea content of rat blood. In their work they used the mixed blood of six normal rats and found 2 mg. of uric acid, 38 mg. of non-protein nitrogen, and 22 mg. of urea per 100 cc. of blood. The urea value expressed as urea nitrogen would be 10.25 mg. Voegtlin, Dunn, and Thompson (5), in 1924, found an average of 113 mg. of sugar per 100 cc. of blood.

In this paper we present the data obtained from the analysis of the blood from twenty-eight normal and three abnormal rats. Because of the small quantity of blood obtainable from a rat, it was impossible to make a complete analysis of the blood of any one rat, so the animals were divided into two groups. With Group I, sugar, non-protein nitrogen, creatinine, and creatine plus creatinine were determined. With Group II urea nitrogen, uric acid, and sodium chloride were determined.

In all the work here reported blood from individual rats was analyzed. To obtain the blood samples the animals were decapitated, care being taken to excite the animals as little as possible. Lithium oxalate was used to prevent clotting. Each animal was later autopsied to note any evidences of disease. The methods used for the determination of sugar, non-protein nitrogen, creatinine, and creatine plus creatinine were those of Folin and Wu (3, 4). Because of the high non-protein nitrogen content, 5 cc.

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TABLE I.

Composition of Rat Blood. Group I.

The composition is expressed in mg. per 100 cc. of blood.

Rat No.	Sex.	Breed.	Age.	Sugar.	Non-protein N.	Creatinine.	Creatine plus creatinine expressed as creatinine.
			mos.				
361	Male.	Pied.	3	125.3	45.2	1.24	6.51
364	"	"	3	132.3	40.0	1.34	6.57
349	Female.	"	4	123.2	44.8	1.32	6.57
336	Male.	Albino.	5	123.1	47.8	1.34	6.52
Average of four rats 3 to 5 mos. old......				126.0	44.5	1.31	6.54
	Male.	Pied.	6	128.2	45.7	1.23	6.62
	"	"	6	127.2	43.7	1.27	6.54
	"	"	6	130.1	47.8	1.23	6.57
	"	Albino.	6	118.0	47.3	1.24	6.64
	"	"	6	116.7	49.1	1.32	6.54
310	"	"	8	126.6	38.6	1.34	6.12
266	"	Pied.	9	112.3	46.5	1.36	6.52
294	"	"	9	124.2	43.2	1.34	6.41
282	"	"	10	124.2	47.8	1.34	6.54
281	"	"	10	110.1	46.5	1.28	6.98
Average of ten rats 6 to 10 mos. old......				121.8	45.6	1.30	6.55
131	Male.	Pied.	13	124.6	43.4	1.22	6.42
145	Female.	"	14	115.2	45.6	1.23	6.12
118	"	"	16	116.7	45.2	1.15	6.00
Average of three rats 13 to 16 mos. old...				118.8	44.6	1.20	6.18
301	Female.	Pied.	7	435.2	55.8	1.42	6.42
191	"	"	12	153.6	49.4	1.24	6.60
146	"	"	12	185.9	58.0	1.28	6.66
Average of three rats with bad lungs.....				261.6	54.4	1.31	6.56
Average of seventeen normal rats......				122.2	45.2	1.28	6.48
" " fourteen " males....				123.1	45.2	1.29	6.54
" " three normal females.......				118.4	45.1	1.23	6.23
" " four albino rats............				121.1	45.7	1.30	6.46
" " thirteen pied "				122.6	45.0	1.27	6.41

of the ammonium sulfate standard solution were used instead of the 3 cc. usually recommended. In the determination of urea nitrogen an aeration-Nesslerization method was employed, 5 cc. of the Folin-Wu filtrate being used for analysis. Uric acid was determined by Benedict's (1) direct method and chlorides by the method of Whitehorn (6).

Table I summarizes the data collected with Group I. The results are arranged in order according to the age of the animals and averages are given for the different age groups, for male and female, and for albino and pied rats. Table II records the results obtained with Group II. Because of the small number of individuals, no attempt has been made to divide them into groups.

TABLE II.

Composition of Rat Blood. Group II.

The composition is expressed in mg. per 100 cc. of blood.

Rat No.	Sex.	Breed.	Age.	Urea N.	Uric acid.	NaCl
			mos.			
4277	Male.	Pied.	3	18.3		
668	"	Albino.	7	16.5	1.39	477.0
	"	Pied.	7	16.3		
	"	"	7	14.3	1.35	502.8
	"	"	7	14.5		
	"	"	7	9.9		
627	"	"	8	15.2	1.92	472.8
599	Female.	"	9	20.6	2.12	550.7
600	Male.	"	9	14.2	2.12	524.0
555	"	"	13	15.2	1.69	507.0
507	Female.	Albino.	16	16.2	2.40	572.0
Average..................................				15.6	1.86	515.2

An inspection of Tables I and II shows that in most respects rat blood is quite similar in composition to human blood. The most noticeable exception is in the case of non-protein nitrogen where the average value is considerably above that for human blood. Blood sugar, urea nitrogen, and chloride values are slightly higher than one finds in normal human blood. In connection with the values given for uric acid it should be noted that these possibly do not represent true values for this constituent since the method used is said by the author to be applicable only to human blood. Whether or not this method is applicable to rat blood remains to be determined.

From Table I, where average values are given for various age groups, male and female, and albino and pied rats, it is evident that there is very little variation in the various groups. Blood sugar values decrease slightly with age and females give a lower average than males.

On autopsy, three rats in Group I were found to have badly infected lungs. Since the blood from these animals showed marked deviations from the normal, with respect to sugar and non-protein nitrogen values, the results are included. The average blood sugar value for this group is over twice that for normal animals. Non-protein nitrogen values are definitely higher than the normal.

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No. 1842, pages 422–423.

THE EFFECT OF COLLOIDAL SILICA ON THE ABSORPTION OF PHOSPHORIC ACID BY PLANTS

REPLYING to a criticism of Fisher[1] concerning the interpretation of the effect of sodium silicate in increasing the assimilation of phosphoric acid by barley in the Rothamsted field experiments, Hall[2] suggests that an explanation of the phenomenon may be sought in the colloidal behavior of the sodium silicate in accordance with the theory advanced by Comber.[3] The latter maintains that the soil colloids play an important rôle by being directly absorbed by plants through the formation of a one phase system between the colloidal coating of the plant root hairs and the colloidal coating of the soil particles. This proposed modification[3] of present views by Comber,[3] however, has been critically discussed by the writer,[4] who has shown that the weight of existing evidence does not support the theory of the absorption of colloids by plants.

There is ample evidence,[5, 6, 7, 8] moreover, to disprove the view that silica may act as a substitute for phosphoric acid in the metabolic processes—it is not "phosphorsäuresparend"; nor can the view[9] that the observed effect is due to the improvement in the physical condition of the soil withstand critical investigation.[10] Relative to the alternative explanations presented by Hall and Morison,[7] certain authorities[1, 6, 8] have concluded from the increased quantity of phosphoric acid per dry weight of the crop that the beneficial effect of silica is due to the increased availability resulting from an increase in the quantity of phosphoric acid in the soil solution. Gile and Smith[6] and also Lemmermann[8] concluded that this action is a solvent one on the soil phosphates. The former[6] substantiate their conclusions from experiments (albeit *in vitro*) which showed a greater solubility of rock phosphate in the presence of silica gel, whereas the latter[8] recognizes that the explanation just advanced is incomplete. The fact, however, that plants can thrive in extremely dilute solutions of phosphate ion if the supply is maintained[11, 12] even as low as 0.25 p.p.m.[13] proves that the solvent effect of silica on soil phosphates, as suggested by these investigators, is a negligible factor compared with the other more powerful solvent forces present.

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[1] R. A. Fisher, *Jour. Agr. Sci.*, 19: 132–139, 1929.

[2] A. D. Hall, *Jour. Agr. Sci.*, 19: 586–588, 1929.

[3] Norman M. Comber, *Jour. Agr. Sci.*, 12: 363–369, 1922.

[4] Walter Thomas, *Soil Sci.*, 27: 249–270, 1929.

[5] Wl. S. Butkewitsch and W. W. Butkewitsch, *Biochem. Ztschr.*, 161: 468–487, 1925.

[6] P. L. Gile and J. G. Smith, *Jour. Agr. Res.*, 31: 247–260, 1925.

[7] A. D. Hall and C. G. T. Morison, *Proc. Roy. Soc.*, B–77: 455–477, 1906.

[8] O. Lemmermann, H. Wiessmann and K. Sammet, *Ztschr. Pflanzenernähr. u. Düngung*, 4A: 265–315, 1925.

[9] Fr. Duchon, *Ztschr. Pflanzenernähr. u. Düngung*. 4A: 316–325, 1925.

The seat of action ultimately must be in the plant itself,[7] for the absorption of ions by the plant is conditioned by the equilibrium conditions prevailing in the interface of the system composed of the ions in the plasma membrane of the root hairs and the ions of the soil solution. Now, in a system containing a non-diffusible ion—$SiO_3^=$ in the case under discussion—a marked effect is exerted on the distribution of diffusible ions on either side of the cell membrane. A non-diffusible electro-negative ion, such as a colloid micelle,[4] will produce a greater concentration of diffusible electro-negative ions and a lower concentration of electro-positive ions on its own side of the membrane than on the other. A non-diffusible electro-positive ion will produce the opposite effect. According to the Gibbs-Donnan distribution law[14, 15] at equilibrium the product of the concentrations of each pair of oppositely charged diffusible ions is the same on both sides of the membrane. Hence, in the system under consideration if x and y represent the molar concentrations of the diffusible H and PO_4 ions, respectively, on each side of the interface considered, and z the concentration of the non-diffusible SiO_3 ions, then, according to the Donnan principle, displacement of the diffusible ions takes place in such a way that at equilibrium $x^2 = y(y+z)$. It follows that the greater the concentration of non-diffusible anions ($SiO_3^=$) present, the greater must be the absorption of diffusible anions ($PO_4^{\equiv}$) into the cell. The equation also shows that x and y are equal only when $z = 0$. Practically, there is a limit to the amount of silica that might be added, because of the coating of the root hairs and other factors.[5] Obviously, the Donnan effect will be restricted by the presence of diffusible $xSiO_2 \cdot yH_2O$ ions, some of which will be present in the solution.[4]

The experiments of Butkewitsch and Butkewitsch[5] with collodion membranes and also with maize plants grown in Pfeiffer's nutrient solution with and without phosphoric acid to which different amounts of silicic acid were added afford the first experimental evidence in support of the interpretation just submitted.

It is realized that the Gibbs-Donnan distribution law may not suffice to explain all the facts connected with the absorption of ions from the soil solution by the plasma membrane of the root hairs of plants, for the Donnan principle applies to free ions only.

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[10] O. Lemmermann, *Ztschr. Pflanzenernähr. u. Düngung*, 4A: 326–330, 1925.

[11] D. R. Hoagland and J. C. Martin, *Soil Sci.*, 16: 367–388, 1923.

[12] F. W. Parker, *Soil Sci.*, 24: 129–146, 1927.

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[15] F. G. Donnan and A. B. Harris, *Jour. Chem. Soc. (Lond.)*, 99: 1554–1557, 1911.

From Wright's investigations[16] on the secretion of the high amounts of calcium in milk from the relatively low concentrations of this element in the blood plasma, it is apparent that the degree of dissociation of the protein salts must be taken into consideration also, since these ions are present in the cell in addition to those which entered to satisfy the product equation. The plant cell is a polyphase system; from this starting-point Briggs and Petrie[17] have deduced that the apparent internal ionic product resulting from the collective effect of all the phases within the tissue in this polyphase system must have a higher value than that of the external medium. It must be kept in mind, however, that deductions from experiments[17,18,19] in isolated tissues or cells of plants, although supplying valuable information, may not necessarily conform to the behavior of the plant as a whole. Herein, from the plant physiology standpoint, lies the value of the type of experiments carried out by Butkewitsch and Butkewitsch.[5]

It remains only to be pointed out that in the Rothamsted experiments sodium silicate, a salt showing a strong tendency to hydrolyze, was used, whereas in the other experiments cited in this paper the silica was added in the form of silicic acid gel. The effect, therefore, of hydrogen-ion concentration on the mobility of ionic pairs must also be considered.[20,21]

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[16] Norman Charles Wright, *Biochem. Jour.*, 23: 352–357, 1929.

[17] G. E. Briggs and A. H. K. Petrie, *Biochem. Jour.*, 22: 1071–1082, 1928.

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65

Ice Cream's Place in the Human Diet

BY R. ADAMS DUTCHER,
Professor of Agricultural Biochemistry, Pennsylvania State College.

From the standpoint of nutritional research, the story of the development of our knowledge regarding the nutritive value of ice cream is naturally the story of the development of our knowledge regarding the nutritive value of milk and its products. It will be necessary for me, therefore, to dwell to a considerable extent on research work which has led us to a more perfect appreciation of the nutritive value of milk.

I should like, therefore, to ask you to go with me, for a few minutes, back to the beginnings of our knowledge of milk and its products. We will watch the chemist and the biochemist gradually unravel some of the knotty problems that have been baffling scientists for many years. When we are able to tear milk apart into all of its components and feed and study these, we will discover why the dairyman and ice cream dealer can well be proud that they are producing and dispensing products of such health producing qualities. As a scientist I have often had difficulty in understanding how salesmen, manufacturers and dealers could enjoy selling many commodities that are sold on the market. I can understand, however, how a man can be most happy, proud and clear of conscience who is in any phase of business which has to do with the production, manufacture or sale of high grade dairy products.

Three Periods of Research

In a general way, there have been three periods in nutritional research, so far as the development of feeding standards for man and beast are concerned. It was natural, of course, that the greatest progress should be made and the beginning of a sound nutritional philosophy should be established as soon as the science of chemistry began to develop. The chemist soon turned his attention to foods and feeding materials and it was not long before he was able to classify foods and feeding materials on the basis of their chemical composition.

In other words, it was possible to classify foods on the basis of their content of water, protein, fat, carbohydrate, fiber and mineral matter. As a result, the nutrition worker seized upon these findings in order that he might devise a measuring stick, in the way of chemical standards, for men and animals. *The first measuring stick, therefore, was a chemical one.* Men and animals of certain sizes and weights should receive certain amounts of protein, fat and carbohydrate daily if normal growth could be expected. These discoveries were most helpful and many very fine contributions to knowledge have been made through the use of such standards.

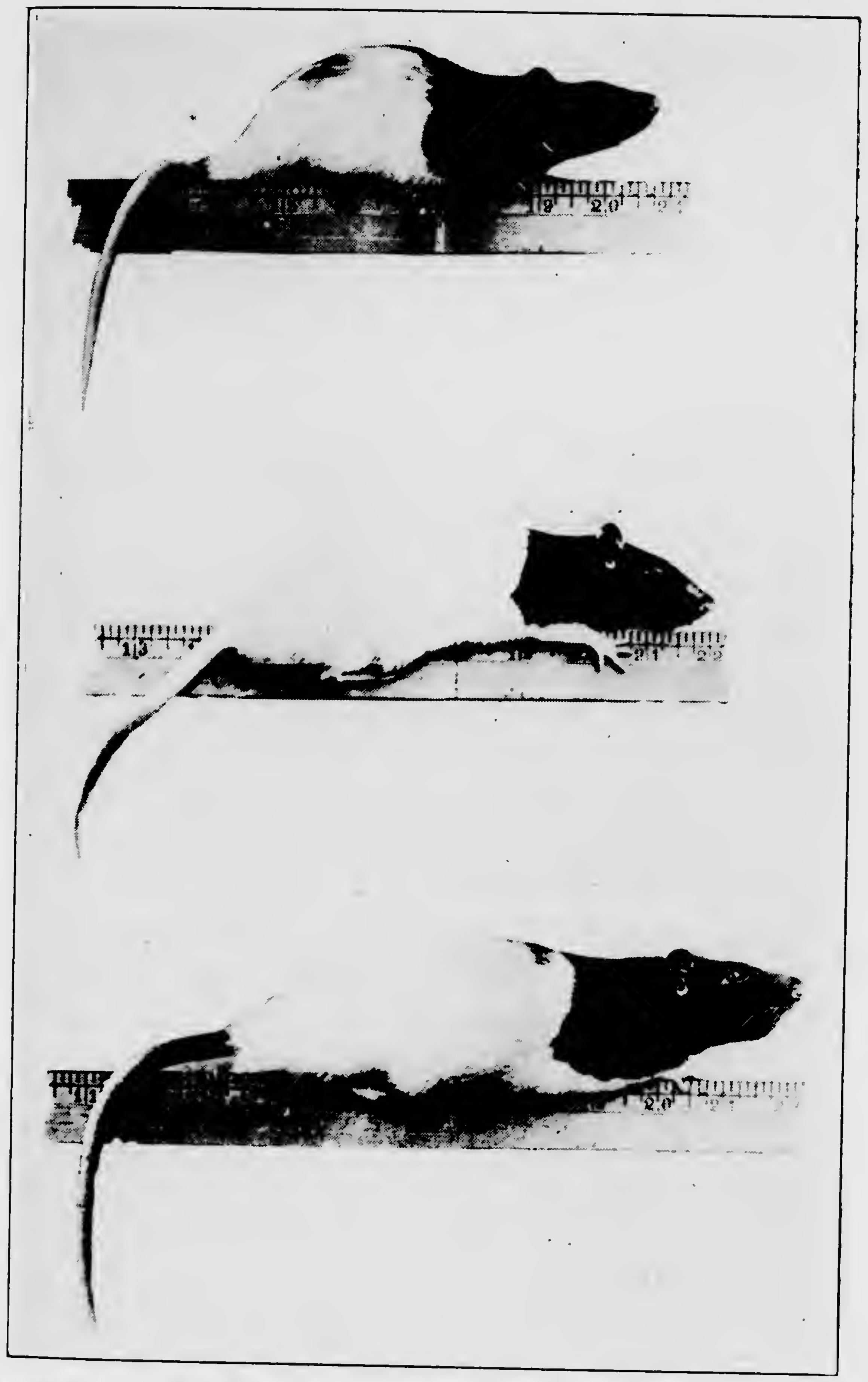
Research work on animal-heat soon led to the development of calorimeters, instruments capable of measuring the amount of animal-heat emitted under various conditions. It was found that definite relationships existed between the amount of energy stored in the food, the amount of food eaten and the energy demands of the body. As a result of this work a new measuring stick was devised which I shall call the "energy" or "calorie" method of measurement. You are all so familiar with the necessity for calories that further mention is unnecessary except to say that the calorie standards did not ignore the chemical constituents, in fact it was recognized that a proportion of the calories must be furnished in the form of protein. These energy standards have been exceedingly useful in establishing fundamental and economic information concerning the feeding of men and animals.

During the past twenty years a new viewpoint has gradually developed, due to the fact that rations can be devised according to the best of chemical and caloric standards on which animals will not thrive, and in many cases diseases, known as "deficiency diseases," develop. This work has brought scientists, medical men and even the layman to appreciate, for the first time, the importance of the "little things in nutrition." Before I discuss this phase of work I wish to show how it really developed.

In 1881 an European investigator by the name of Lunin described experiments in which purified diets were devised according to the best of scientific knowledge of the period. Mice which received these diets did not grow. The addition of a small amount of milk, however, caused growth to be resumed.

Eijkmann, a Dutch physician, published a paper in 1897 in which he described experiments with fowls which had developed a paralytic disease, called beri-beri, on a diet of polished (white) rice. He found that the brown outer coating of the rice (bran) prevented and cured the disease and he was able to extract the curative material from the polishings by means of alcohol and water. He concluded that white rice contained a poison or toxin and that the rice bran possessed an anti-toxin-like substance.

In 1906 Dr. F. G. Hopkins of England repeated the experiments of Lunin and confirmed the latter's results. He found that two cubic centimeters of cow's milk contained something which



The results of Dr. L. S. Palmer's work on the nutritive value of ice cream, showing that ice cream may supplement the ordinary diet advantageously.

The top rat fed basic diet alone. Note size of rat and condition of eye. Middle rat fed ice cream exclusively. Note size and physical condition. Bottom rat fed basic diet plus ice cream. Note size and physical condition.

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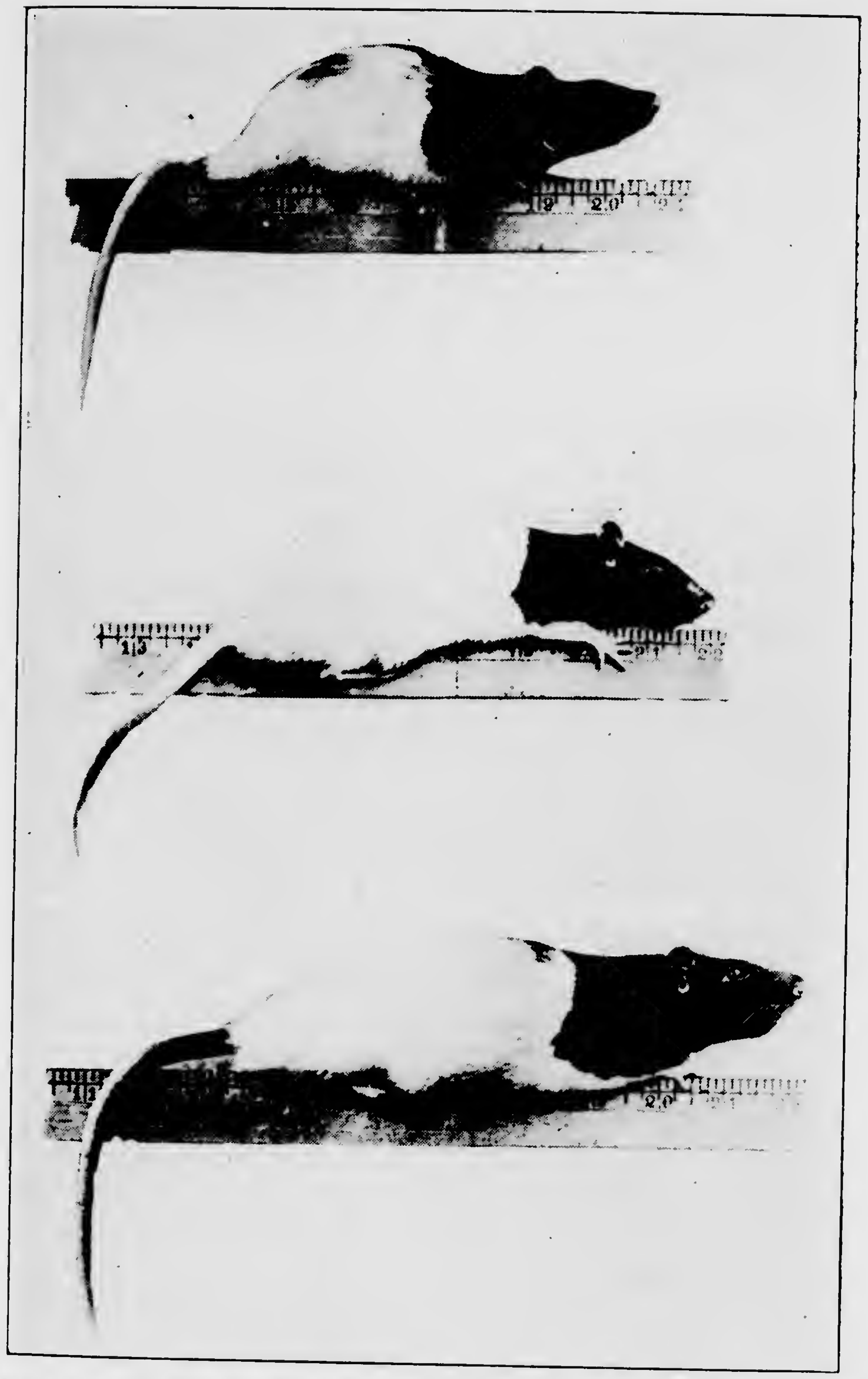


The results of Dr. L. S. Palmer's work on the nutritive value of ice cream, showing that ice cream may supplement the ordinary diet advantageously.

The top rat fed basic diet alone. Note size of rat and condition of eye. Middle rat fed ice cream exclusively. Note size and physical condition. Bottom rat fed basic diet plus ice cream. Note size and physical condition.

was necessary for growth, which was not a protein, a fat, a carbohydrate, or a salt. In other words, he postulated that milk contains an "accessory food factor."

Vitamins Enter the Picture

It was not until 1910-12 that Dr. Casimir Funk, a Polish chemist, substantiated the work of Eijkmann using extracts of rice bran, from which he isolated a crystalline material which seemed to be curative in beri-beri, to which he gave the name "vitamine." At the time he prophesied that many so-called dietary deficiency diseases would prove, eventually, to be "vitamine deficiency" diseases.

In the meantime, small animal experimentation was being started at Wisconsin by McCollum and coworkers and at Yale by Osborne and Mendel. The outcome of this work was of the greatest importance to the dairy industry. In casting about for ideal purified proteins, fats, carbohydrates and salts three important discoveries were made, i. e., (1) that all proteins did not possess the same growth promoting properties, (2) that the mineral elements deserved greater attention than they had received heretofore, and (3) that the vitamin hypothesis of Funk was confirmed in a very definite manner.

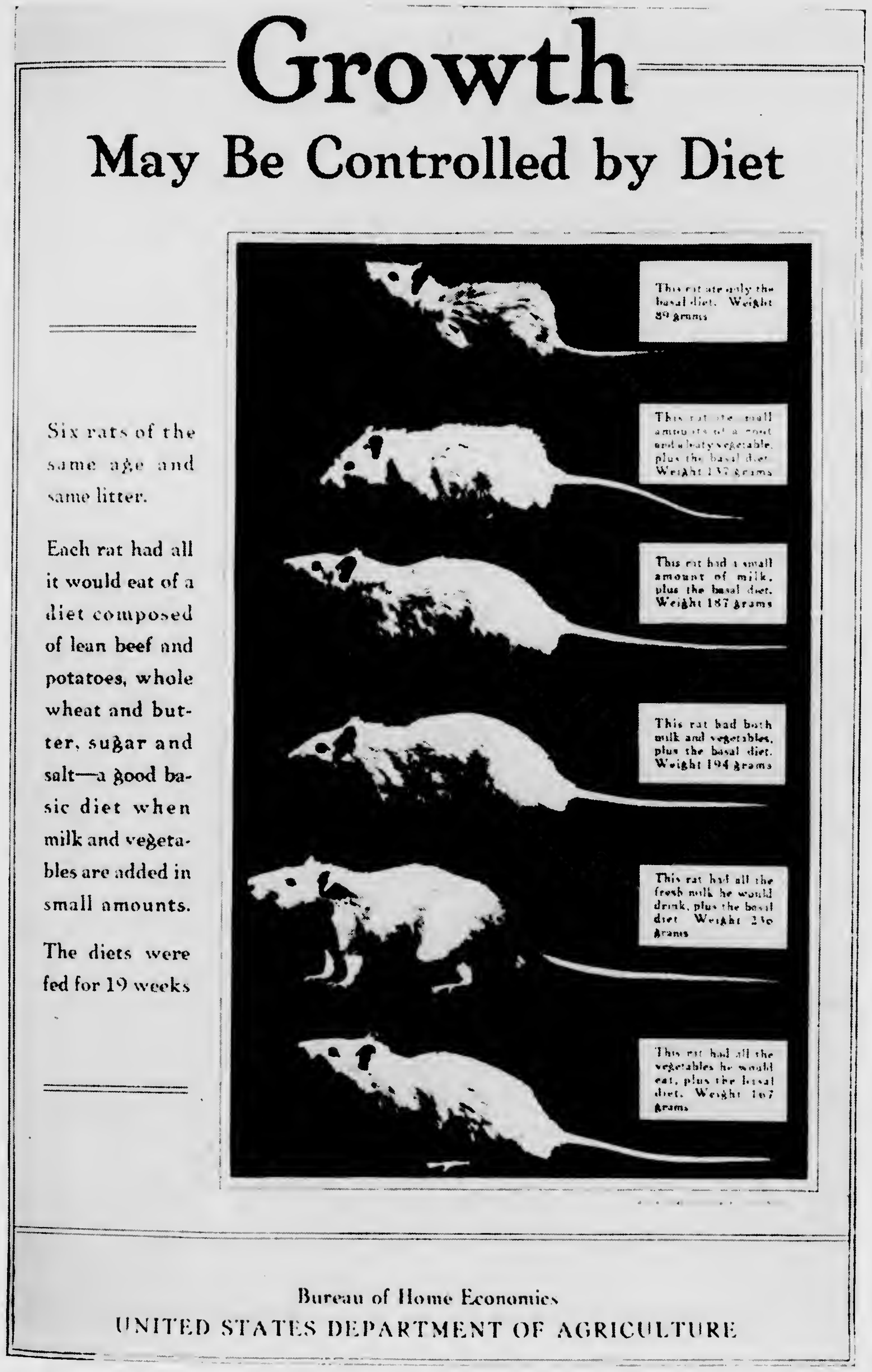
Time and space will not permit me to dwell on these interesting researches except to say that the superiority of animal proteins, particularly casein and lactalbumin of milk was established by Osborne and Mendel. Further study of the question revealed the fact that casein, lactalbumin and other biologically superior proteins contained certain amino acids in kind and amount not present in the inferior proteins. Thus, the dairy man and dairy technologist found one reason why milk and its products had always been of superior nutritive value.

It was found also that the best artificial salt mixtures were those that were patterned most closely after the mineral composition of milk. Later work has shown that calcium and phosphorus are present in milk in particularly advantageous proportion and quantity.

Almost simultaneously the Wisconsin and Yale investigators discovered the presence of a fat-soluble vitamin in butterfat, cod liver oil and egg yolk. It was not present in most plant fats and oils. Hence, another most important function of milk and milk products was added to the already growing list of nutritional factors.

Different Vitamins Described

McCollum and Kennedy suggested that these accessory factors be given letters until such a time as they could receive chemical names. The fat-soluble A of McCollum and Kennedy is



Growth

May Be Controlled by Diet

Six rats of the same age and same litter.

Each rat had all it would eat of a diet composed of lean beef and potatoes, whole wheat and butter, sugar and salt—a good basic diet when milk and vegetables are added in small amounts.

The diets were fed for 19 weeks

This rat ate only the basal diet. Weight 89 grams

This rat ate small amounts of a root and a leafy vegetable, plus the basal diet. Weight 137 grams

This rat had a small amount of milk, plus the basal diet. Weight 187 grams

This rat had both milk and vegetables, plus the basal diet. Weight 194 grams

This rat had all the fresh milk he would drink, plus the basal diet. Weight 236 grams

This rat had all the vegetables he would eat, plus the basal diet. Weight 167 grams

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A demonstration of the value of supplementing ordinary diets with vegetables and milk.

Ice cream is milk in concentrated form.

now known as "Vitamin A." At about the same time McCollum and Kennedy found that Funk's anti-beri-beri factor was also needed for growth. As a result they suggested the name "Water-soluble B" which is now known as "Vitamin B." At that time the word "vitamine" was spelled with an "e." The letter "e" was dropped a few years ago and the word is now spelled "Vitamin." The first syllable is pronounced as in the word "vital" and the last syllable is pronounced the same as the name of Andy Gump's wife. The accent, I might add, is on the first syllable.

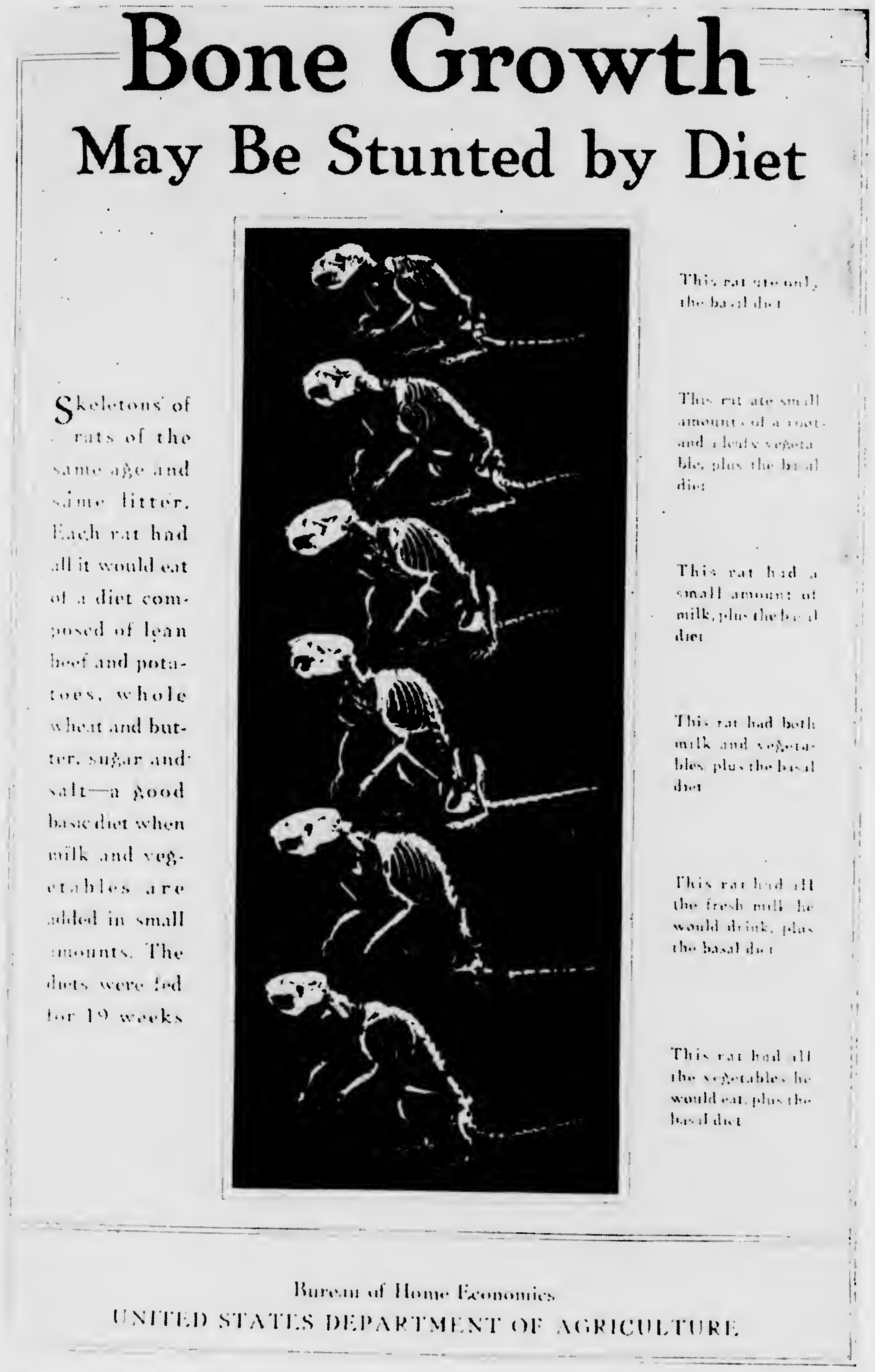
Recently Vitamin B has been found to consist of two vitamins, i. e., a heat labile anti-beri-beri fraction and a heat-stable appetite promoting and pellagra-curing fraction. The American workers tend to restrict the term "Vitamin B" to the former and have given the latter the name "Vitamin G." The British workers have coined the terms B_1 and B_2 respectively, to refer to these vitamins. Milk contains these vitamins also, Vitamin G (B_2) being present in the greater amount.

Vitamin C, the vitamin which cures scurvy, was discovered next. This vitamin was also found to be a constituent of normal milks. Our own research work and that of others has shown that Vitamins A and C fluctuate in cow's milk, depending on the vitamin content of the diet of the cow. Under most conditions I should say that milk cannot be considered to be rich in Vitamin C, although the amount present is often appreciable.

Vitamin D, the vitamin which has to do with the utilization of calcium and phosphorus and proper bone and teeth formation, was identified by McCollum and coworkers. While this vitamin is also present in milk, it is never present in large amounts. Cod liver oil remains the richest natural source of this vitamin. A few years ago it was discovered that ultra-violet light produced results similar to Vitamin D, when allowed to shine on animals or children afflicted with rickets. Later, Hess, of New York, and Steenbock, of Wisconsin, discovered that Vitamin D could be manufactured in certain food materials by irradiating the food material with ultra-violet light. More recently an alcohol, called "ergosterol," has been isolated which is considered to be the parent substance of Vitamin D. When this alcohol is treated with ultra-violet light it becomes many thousand times more potent than cod liver oil, in the cure of rickets.

Vitamin E, the anti-sterility factor, was discovered by Dr. Evans and coworkers of California. This vitamin cures and prevents nutritional sterility in experimental animals. While milk and butter are extremely deficient in this vitamin, it does not appear to be a matter of concern for the reason that it is well distributed in other foods.

Vitamin F, which was thought to exist, is not recognized at present. This leaves a gap in our alphabetical scheme as recommended by American workers.



Bone Growth

May Be Stunted by Diet

Skeletons of rats of the same age and same litter. Each rat had all it would eat of a diet composed of lean beef and potatoes, whole wheat and butter, sugar and salt—a good basic diet when milk and vegetables are added in small amounts. The diets were fed for 19 weeks

This rat ate only the basal diet

This rat ate small amounts of a root and a leafy vegetable, plus the basal diet

This rat had a small amount of milk, plus the basal diet

This rat had both milk and vegetables, plus the basal diet

This rat had all the fresh milk he would drink, plus the basal diet

This rat had all the vegetables he would eat, plus the basal diet

Bureau of Home Economics
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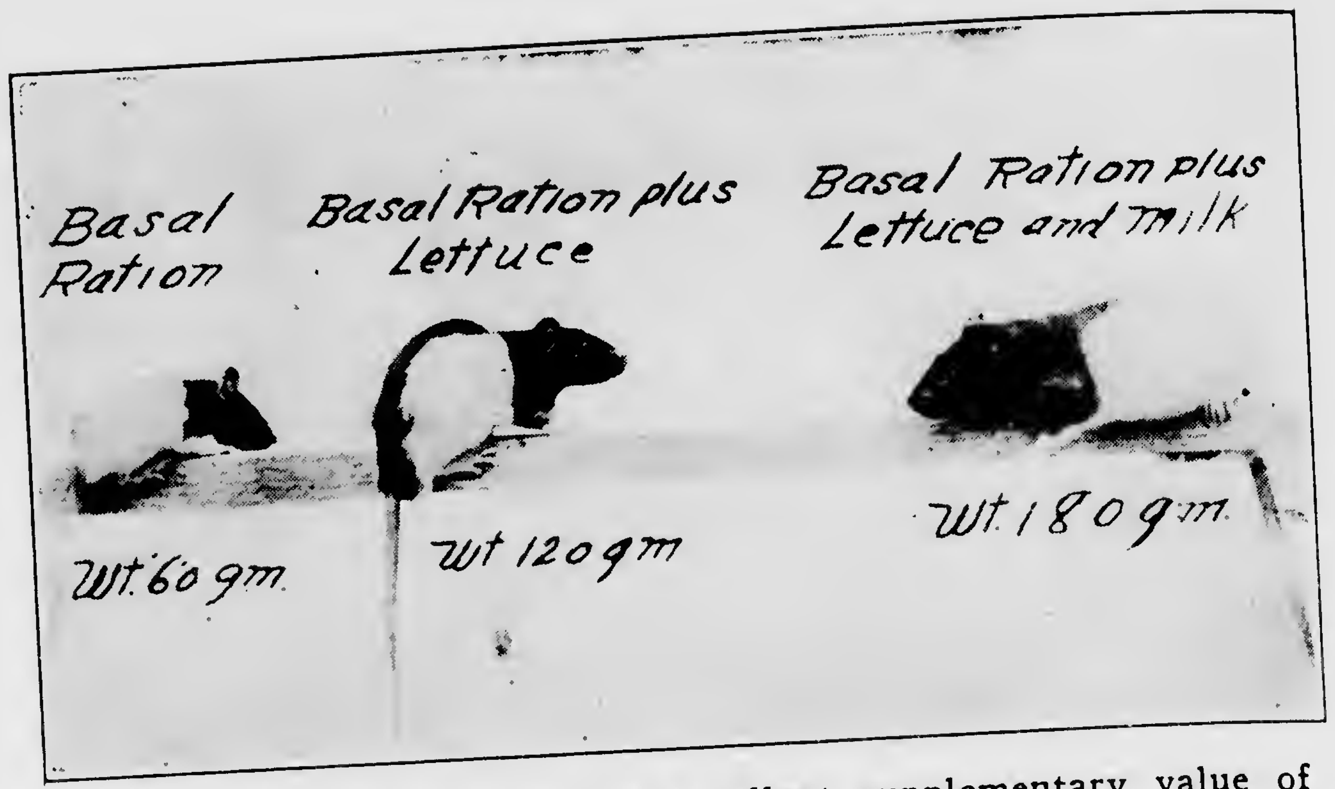
Showing the effect of vegetables and milk on skeletal development.

Mineral content of ice cream is approximately 30% higher than that of milk.



Further evidence to show the excellent supplementary value of milk solids.

The milk solids content of ice cream ranges from 18 to 22 per cent compared with 12 per cent in milk.

Vitamin G, the appetite, growth and anti-pellagra factor, is present in normal milks in appreciable amounts.

Our own work has shown that milk will be relatively rich in these health producing factors if the cow has been properly housed and fed. Our researches on pasteurization, drying and evaporating show quite clearly that these operations can be conducted without alarming amounts of vitamin destruction. In coöperation with Dr. S. I. Bechdel and Professor M. H. Knutsen we have shown that the Vitamin B complex in cow's milk is not completely dependent on the ration and we have shown that she can manufacture this complex in her rumen by means of certain bacteria.

With this information at hand allow me to ask what might be expected if we took condensed whole milk, cream and sugar and made a typical ice cream mix consisting of about 40 per cent solids. The previous research work I have described has shown that we have, in milk proteins of superior biological value, minerals of the type that nature demands and, in the butter fat, an abundance of Vitamin A which is necessary for growth and good health.

Importance of Vitamin A

If I may be allowed to digress at this point I wish to emphasize the great importance of Vitamin A in the diet. When this vitamin is omitted from the dietary a number of important pathological changes occur. Its absence is made manifest by loss of appetite and a resulting loss of body weight. An eye disease, known as xerophthalmia, develops which afflicts rats and humans alike. The resistance to bacterial infections is lowered and respiratory diseases invariably ensue. Sinus and tongue infections are common and we have noted involvement of the kidneys and reproductive organs. Physicians are able to apply the results of such findings to the treatment of human respiratory disease. I have been informed by eminent laryngologists that they have been able to alleviate many cases of sinus and respiratory infections by combining Vitamin A therapy, through the use of cream, butter fat and cod liver oil, with their regular medical treatment.

I think I have said enough about the contribution that milk, cream and butter fat products make toward better health and growth to convince you that we can prophesy what well made ice creams would do when used as a supplement to most diets.

Ice Cream, A Food *Par Excellence*

You are already familiar with the experiments of Dr. L. S. Palmer, conducted in the Forest Lake Laboratory of the National Dairy Council. Dr. Palmer showed that the ordinary diet of bread, meat, potato and cereals was supplemented most advan-

A case of human xeropthalmia due to insufficient Vitamin A. Note the child with patch over left eye. These children were forced to subsist on cereal soups during the World War owing to the fact that all dairy live stock in Roumania had been commandeered by the Austrians.

Ice cream is rich in Vitamin A through its butterfat content.

Dr. S. I. Bechdel and Dr. J. F. Shigley of the Pennsylvania State College, with Penn State Jessie. Jessie obtained considerable publicity owing to the fact that she had a permanent opening in her left side from which her food could be removed and studied. It was found that she could synthesize her own Vitamin B by means of bacteria which grew and flourished in her rumen.

tageously by ice cream. Animals receiving the basal diet of meat, bread, potatoes and cereals grew less satisfactorily. Even when the diet consisted of ice cream alone, the body weight exceeded that of the animals which received the basal diet.

Dr. Homberger's reports from Kentucky confirm the findings of Dr. Palmer.* The detailed results of his work will be looked forward to with interest by scientists and the general public.

The widespread use of ice cream by hospitals in the diets of convalescents shows, in a most practical way, that ice cream must be a food *par excellence* in the minds of medical men—so far as palatability, digestability and nutritive value are concerned.

In closing, may I add a word of congratulation and advice. You are to be congratulated upon the foresight and vision to organize and develop this wonderful clearing house for ideas in promoting the best interests of your members. It is, in my opinion, a most healthy sign, when competitors are able to forget trade secrets, competition and similar things and sit down together to give the best of their own ideas and experiences for the good of the industry.

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*"Nutritive Value of Ice Cream," by Dr. L. S. Palmer, *American Journal of Public Health*, Vol. XIX, No. 6, June, 1929.

My advice to you is to continue to develop this spirit of coöperation and to support, with your time and money, research in all its phases. Industry in general, and your own industry in particular, will progress much faster with a well-planned, long-time research program. It has been amply demonstrated that profits and opportunity for human service come first to those who, through the findings of scientific research, are ready to take advantage of new situations when opportunity knocks at the door.

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STUDIES ON THE CHEMICAL COMPOSITION OF BOVINE BLOOD*

ARTHUR K. ANDERSON, HOWARD E. GAYLEY, and AVERY D. PRATT

From the Department of Agricultural and Biological Chemistry of The Pennsylvania State College, State College, Pennsylvania

With the development of methods for the analysis of blood, a valuable tool has been placed in the hands of experimenters in physiology and nutrition and of physicians in the clinical laboratory. At the present time a great deal of information is available on the abnormal composition of human blood in disease. A knowledge of the composition of normal blood was obviously a preliminary necessity for the interpretations of these abnormalities.

In order to extend the use of blood analysis to cattle, normal values must first be established for the various blood constituents. A considerable amount of data along this line are available at the present time. Abderhalden (1) in 1898 published a rather complete analysis of the blood of various domestic animals including cattle. More recently important contributions have been made by Meigs, Blatherwick and Cary (22), Blatherwick (6), Robinson and Huffman (23) and Hayden and his co-workers (18, 19, 20). In the course of our study on the composition of the blood of dairy cattle under various conditions we have accumulated considerable data on normal animals which we have assembled in this paper. It is hoped that these data may add to the present knowledge of normal bovine blood and may be of value to future workers in the field of bovine physiology, nutrition, and pathology.

METHODS

Blood samples were obtained from the jugular vein with as little stasis as possible. Two samples were always taken, one in a small Erlenmeyer flask, with no oxalate, for the preparation of serum in which calcium and phosphorus were determined; the other in a large test tube under paraffin oil, using 40 mgm. of lithium oxalate to prevent clotting. About 75 cc. of blood were collected in this manner to be used for the determination of hemoglobin, carbon dioxide and the substances included in the Folin-Wu system of blood analysis. The portion of the sample used for the determination of carbon dioxide was centrifuged immediately under paraffin oil and the plasma removed.

Hemoglobin and carbon dioxide were determined by the gasometric methods of Van Slyke (25, 26). For the estimation of non-protein nitrogen, creatine, creatinine, uric acid, sugar and chlorides, a protein free filtrate was prepared according to the procedure of Folin and Wu (12). Non-protein nitrogen, creatine and creatinine were determined by the original procedure of Folin and Wu (12). Uric acid was determined by Benedict's method (5), sugar by the method of Folin and Wu (13) and chlorides by the method of Whitehorn (28). Urea nitrogen was determined on whole blood by the method of Van Slyke and Cullen (27). Phosphorus was determined in three forms, namely, inorganic, hydrolyzable organic and total acid soluble phosphorus, using the Briggs modification (7) of the Bell-Doisy method (2) with some slight modifications of procedure. Calcium was determined by Clark and Collip's modification (8) of the Kramer and Tisdall method (21, 24).

EXPERIMENTAL

The work embodied in this report includes the analysis of 59 samples of blood taken from the dairy herd of the Pennsylvania State College. The animals include calves and adults of various ages and breeds. The animals received the regular ration used for the dairy herd. The blood samples were taken during the late summer, fall, and winter and no attempt was made to study seasonal variations. The results, which are averages of duplicate determinations, are presented in tables 1 and 2. The animals are grouped into 4 groups on the basis of age. In the first group are those animals less than one month of age. In the second group

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TABLE 1

Showing the composition of the blood of normal dairy cattle

ANIMAL NUMBER	SEX*	BREED*	AGE*	WHOLE BLOOD								SERUM				PLASMA
												Phosphorus				
				Hemoglobin	Non-protein nitrogen	Urea nitrogen	Uric acid	Creatine	Creatinine	Sugar	Chlorine as sodium chloride	Inorganic	Inorganic plus hydrolyzable organic	Total acid soluble	Calcium	Plasma bicarbonate CO_2
Animals ranging from 1 to 28 days of age																
				per cent normal	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*volumes per cent*
1312	M.	A.	0– 0– 1		30.80	4.40	1.97	4.81	1.62	123.0	503				12.93	
1308	M.	H.	0– 0– 6		25.91		1.59	4.67	1.55	96.0	484					
1310	M.	H.	0– 0– 7		35.42	15.63	2.40	4.42	1.82	89.0	473				13.40	
1323	F.	A.	0– 0– 9	121.7	42.14		3.10	4.06	1.49	105.7	457	5.23	5.72	7.80	12.52	52.2
1327	F.	H.	0– 0–10	104.0	26.99	19.80	1.53	3.83	1.73	99.8	440	5.05	7.60	8.44	13.46	58.9
1321	F.	A.	0– 0–11	90.8	37.15	8.21	2.19	3.35	1.47	98.4	471	4.55	5.75	6.05	11.76	62.6
1309	M.	A.	0– 0–13		31.55	10.53	2.13	5.57	1.41	105.0	492				12.88	
1307	M.	H.	0– 0–16		30.77	10.00	1.76	4.07	1.63	138.0	505				12.60	
1324	F.	G.	0– 0–17	99.2	20.67	12.68	1.71	3.65	1.54	86.8	426	5.30	7.90	8.57	15.00	65.5
1323	F.	A.	0– 0–28	96.7	26.97	15.10	1.91	3.33	1.57	98.9	434	5.20	6.45	8.03	14.48	62.6
Animals ranging from 1 to 5 months of age																
1321	F.	A.	0– 1– 7	79.8	27.62	15.59	1.64	4.78	1.61	104.5	479	6.17	7.50	8.47	14.49	65.5
1308	M.	H.	0– 1–29		29.92	8.62	2.21	4.27	1.38	81.6	515	3.52			12.87	64.5
1000	M.	H.	0– 2–		26.64	14.05	2.86	2.49	1.53	64.3	536					61.7
1168	F.	H.	0– 2–	89.9	33.38	6.21	2.96	4.08	1.52	119.5	448	5.36	6.85	8.24	16.18	64.5
1169	F.	H.	0– 2–	105.4	34.47	12.99	2.00	4.95	1.60	142.8	460	4.77	6.57	7.59	13.81	56.0
1182	M.	H.	0– 2–	85.4	36.82	11.30	1.70	4.58	1.94	138.6	506				11.19	76.8
1168	F.	H.	0– 2–	98.5	31.86	7.12	1.94	6.99	1.45	90.5	487	5.72	7.70	7.91	12.64	65.3
1169	F.	H.	0– 2–	100.9	30.88	10.08	2.00	6.86	1.28	100.0	514	5.15	7.08	7.77	11.49	58.7
1170	F.	H.	0– 2–	95.1	31.58	7.57	1.85	5.83	1.60	120.7	461	3.95	6.28	7.07	11.59	73.0
1307	M.	H.	0– 2– 2		25.73	7.99	1.55	4.32	1.36	71.7	505	3.09			11.99	59.8
1296	M.	A.	0– 2– 2		30.52	12.79	2.00	4.25	1.27	77.0	501				14.30	
1292	F.	J.	0– 2–14	70.3	29.21		1.56	4.48	1.43	73.0	495				12.62	65.5
1302	M.	H.	0– 2–15		27.90	18.91	1.70	4.01	1.42	78.3	546	3.29	3.47		12.96	71.8
1303	M.	H.	0– 2–15		26.38	21.64	1.63	4.37	1.42	77.7	546	3.32	3.46		13.31	67.1
1301	F.	H.	0– 2–21		26.83	13.55	2.08	4.34	1.37	119.9	492	3.41			11.61	67.3
1293	F.	A.	0– 2–22	79.4	29.30	14.30	2.20	7.78	1.45	87.2	540				12.60	52.5
1291	M.	J.	0– 2–25	93.6	26.52	11.70	1.54	3.67	1.28	82.0	495					58.6
1292	F.	J.	0– 2–28		25.00		1.90	4.15	1.25	71.0	506				12.24	
1293	F.	A.	0– 2–28		23.60		1.92	3.67	1.41	80.0	510				10.00	
1290	F.	A.	0– 3– 6	73.3	22.20		1.80	3.89	1.32	72.2	499				11.62	55.1
1288	M.	A.	0– 3–14	86.9	28.00	11.20	1.95	3.61	1.35	75.0	490				10.55	56.7
1287	M.	A.	0– 3–18	86.9	30.18	12.41	1.85	3.83	1.36	67.8	487				12.20	59.8
1290	F.	A.	0– 3–23		25.75	11.00	2.05	2.90	1.19	93.0	513					
1291	M.	J.	0– 4– 5		25.00	10.43	1.96	3.90	1.33	87.3	470				13.80	
1287	M.	A.	0– 4– 8		24.96	11.10	2.05	4.54	1.31	69.0	488				14.16	
1283	F.	A.	0– 4– 8	77.1	27.96	11.99	1.65	3.99	1.32	71.8	496				11.00	63.6
1280	M.	J.	0– 4–15	81.4	26.91		2.20	4.25	1.45	75.0	491				9.96	64.3
1283	F.	A.	0– 4–22		27.48	13.40	1.68	4.47	1.25	66.0	495				11.70	
1280	M.	J.	0– 5– 5		27.53	10.00	1.45	4.04	1.21	82.2	488				11.76	
Animals ranging from 6 to 10 months of age																
1292	F.	J.	0– 6–12	101.2	33.06	13.93	3.22	4.42	1.38	59.6	506	5.45	6.70	7.50	12.55	54.1
1271	F.	G.	0– 6–16	86.2	34.40	15.00	1.71	7.11	1.33	65.5	511				12.20	57.5
1280	M.	J.	0– 6–17		29.13	14.28	1.72	4.06	1.36	90.0	506	3.50			13.40	61.7

TABLE 1—*Concluded*

ANIMAL NUMBER	SEX*	BREED*	AGE*	WHOLE BLOOD								SERUM				PLASMA
												Phosphorus				
				Hemoglobin	Non-protein nitrogen	Urea nitrogen	Uric acid	Creatine	Creatinine	Sugar	Chlorine as sodium chloride	Inorganic	Inorganic plus hydrolyzable organic	Total acid soluble	Calcium	Plasma bicarbonate CO_2
Animals ranging from 6 to 10 months of age—Concluded																
				per cent normal	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*volumes per cent*
1271	F.	G.	0– 6–27		31.90	19.07	2.13	3.56	1.26	78.0	482				11.76	
1266	F.	J.	0– 8– 7		26.43	14.65	1.80	3.89	1.24	67.0	458				11.65	
1265	F.	H.	0– 8–10		32.43	16.42	1.62	3.82	1.26	76.5	481				12.80	
1325	F.	H.	0– 8–10	110.2	31.45	10.08	2.32	4.54	1.51	127.0					14.70	38.1
1266	F.	J.	0– 8–14		35.72	20.00	2.10	2.98	1.30	75.0	491				12.46	
1259	F.	G.	0– 8–19	102.1	42.10	18.00	1.92	4.77	1.39	67.0	480				11.83	61.6
1254	F.	J.	0– 8–29		26.78	13.90	1.52	3.72	1.21	69.6	471				12.60	
1259	F.	G.	0– 9– 0		35.20	17.00	2.10		1.38		478				12.87	
1254	F.	G.	0– 9– 6		35.98	17.93	1.91	3.41	1.32	70.0	476				12.06	
1250	F.	H.	0– 9–18		32.93	14.50	1.50	3.77	1.30	73.8	528				11.50	
1250	F.	H.	0–10– 2		25.60	13.97	1.90	4.08	1.45	74.0	510				11.60	
Animals ranging from 1 to 9 years of age																
1237	F.	G.	1– 0– 4		29.20		2.28	4.05	1.41	68.4	515				12.87	
1233	F.	J.	4– 2–27	81.1	34.20	13.30	2.40	3.83	1.37	51.0	509	3.75	5.10	5.75	13.40	56.0
1233	F.	J.	4– 3–25	109.0	32.20	13.52	2.49	5.01	1.66	47.0					14.78	38.1
1011	F.	B.S. + J.	4– 9–19	91.8	35.76	9.18	2.20	2.80	1.48	45.7	532	3.50	3.90	5.30	12.46	55.1
1011	F.	B.S. + J.	4–11–17	92.4	32.20		2.47	4.11	1.65	43.2					13.49	31.5
838	F.	J.	9– 3–10	103.6	29.23	10.26	2.92	3.79	1.31	51.8					12.88	36.2

* M. = male; F. = female; A. = Ayrshire; H. = Holstein; G. = Guernsey; B.S. = Brown Swiss; J. = Jersey. The final numbers refer to age in years, months and days.

are animals from one to five months of age. The third group includes those from six to ten months of age, and the last group includes animals from one to nine years of age.

In table 2 is given a summary of results given in table 1, with averages for various age groups, for male and female, and for the different breeds. The results obtained by other investigators are also given in this table. The results of other investigators have been recalculated in some cases so that all the data presented in table 2 might be comparable.

Hemoglobin. Tables 1 and 2 show the results of 29 analyses of blood for hemoglobin. The results are expressed as per cent normal and the values range from 70.3 to 121.7, the average value being 92.9. The hemoglobin content appears to be highest in the animals less than one month of age. Females show a higher value than males and Holsteins show the highest average of the various breeds. Our results agree well with those of Hayden and Fish (18).

Non-protein nitrogen. Our figures for non-protein nitrogen are in very close agreement with those of other investigators. Fifty-nine determinations give an average of 30.07 mgm. per 100 cc. of blood with values ranging from 20.67 to 42.14 mgm. The averages for the various groups of animals are all very close to the total average.

Urea nitrogen. Fifty estimations in the course of our work have given an average of 12.94 mgm. of urea nitrogen per 100 cc. of blood, with minimum and maximum values of 4.40 and 21.64 mgm. respectively. The low value of 4.40 mgm. was obtained on a sample from a calf one day old. The averages for the various groups differ only slightly from the grand average except in the case of the Brown Swiss-Jersey cross. It should be noted that this value of 9.18 is for one individual. Our results agree with those of most of the other investigators.

Uric acid. For 59 determinations of uric acid, using the direct method of Benedict (5), we report an average of 2.08 mgm. which exactly duplicates the findings of Hayden and Sholl (19). Our values range from 1.50 to 3.22 mgm. The only group of animals which differs decidedly from this value is that of the older animals,

TABLE 2

Showing a summary of the data given in table 1 and also the results obtained by other workers

Description	WHOLE BLOOD								SERUM				PLASMA
									Phosphorus				
	Hemoglobin	Non-protein nitrogen	Urea nitrogen	Uric acid	Creatine	Creatinine	Sugar	Chlorine as sodium chloride	Inorganic	Inorganic plus hydrolyzable organic	Total acid soluble	Calcium	Plasma bicarbonate CO_2
	per cent normal	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*Volumes per cent*
Average for all animals	92.9	30.07	12.94	2.08	4.30	1.42	84.1	492	4.46	6.12	7.46	12.63	58.9
Maximum for all animals	121.7	42.14	21.64	3.22	7.78	1.94	142.8	546	6.17	7.90	8.57	16.18	76.8
Minimum for all animals	73.3	20.67	4.40	1.50	2.49	1.19	43.2	426	3.09	3.46	5.30	9.96	31.5
Average for animals less than 1 month of age	102.5	30.83	12.04	2.03	4.16	1.58	105.0	469	5.06	6.68	7.78	13.22	60.4
Average for animals from 1 to 5 months of age	86.9	28.28	11.91	1.91	4.49	1.40	87.6	499	4.54	6.11	7.84	12.40	63.3
Average for animals from 6 to 10 months of age	99.9	32.36	15.62	1.96	4.15	1.33	76.4	491	4.48	6.70	7.50	12.42	54.6
Average for animals from 1 to 9 years of age	95.6	32.13	11.56	2.46	3.93	1.48	51.2	519	3.62	4.50	5.52	13.31	43.4
Average for males	86.8	28.83	12.07	1.91	4.19	1.45	89.4	501	3.34	3.47		12.62	63.9
Average for females	93.7	30.71	14.19	2.05	4.34	1.41	81.9	487	4.92	6.51	7.46	12.65	56.7
Average for Aryshires	89.1	29.14	11.69	2.01	4.30	1.40	87.2	491	5.47	6.26	7.59	12.48	59.0
Average for Holsteins	98.7	30.19	10.73	1.96	4.51	1.51	99.2	496	4.24	6.13	7.84	12.73	63.1
Average for Guernseys	95.8	32.78	16.61	1.96	4.63	1.38	72.6	481	5.30		8.57	12.66	61.5
Average for Jerseys	91.5	29.06	13.27	2.06	4.01	1.34	70.1	491	4.23	6.57	6.63	12.62	54.3
Average Brown Swiss-Jerseys	92.1	33.98	9.18	2.34	3.41	1.57	44.5	532	3.50	3.90	5.30	12.98	43.3
Hayden and Fish (18)	89.24	29.79	12.21	2.04 0.623*		1.20	46.52	444.1	5.7		9.29	10.88	
Folin and Dennis (10)		24.00	14.00	0.2*									
Greenwald (14)		32.00											
Hayden and Tubangui (20)		31.00	18.8	2.12		1.84	96.00						
Hayden and Sholl (19)		30.11	11.8	2.08		1.37	51.75	516.1					
Benedict (4)				0.5*									
Folin and Dennis (11)					10.44	2.00							
Greenwald and McGuire (15)					7.23 2.35†	2.58 1.61†							
Abderhalden (1)								507.6	8.47			11.94	
Robinson and Huffman (23)								542.8	5.87			11.0	59.2
Hart, et al. (16)									3.94			16.25	
Meigs, Blatherwick, and Cary (22)									5.38			10.38	
Hart, Steenbock, and Humphrey (17)												10.00	
Blatherwick (6)													61.5‡ 73.0§

* Indirect method.

† Their own method.

‡ Cows.

§ Calves.

from one to nine years of age, where an average of 2.46 mgm. was found.

Our results check well with those of investigators who have used the direct method for the determination of this constituent. Folin and Dennis (10), using a method in which the uric acid is isolated from a blood filtrate as the silver salt before being determined colorimetrically, report a much lower value of 0.2 mgm. Benedict (4) using his modifications (3) of this technique obtained a value of 0.5 mgm. He pointed out that the uric acid determined by this method is "free" uric acid and that this is only a small fraction of the uric acid present. The remainder exists in a "combined" form from which it is easily set free by hydrolysis with acid. Davis and Benedict (9) have shown that the uric acid is combined with a pentose. After hydrolysis of a protein-free blood filtrate, he obtained values as high as 6.7 mgm. It should therefore be pointed out that our results and those of the other investigators whose results are reported in table 2 very likely do not represent true uric acid values but only that which is set free under the conditions of the analysis.

Creatine. In our work, we have found an average of 4.30 mgm. of creatine per 100 cc., with values ranging from 2.49 to 7.78 mgm. The oldest animal group showed the greatest variation from the average, with an average value of 3.93 mgm. The low value of 3.46 for the Brown Swiss-Jersey cross is possibly not significant since there is only one individual in this group. It should be noted that we have reported actual creatine values and not creatine plus creatinine.

Our results do not agree with those of other investigators. Folin and Dennis (11) report 10.44 mgm. and Greenwald and McGuire (15), using Folin's technique, report 7.23 mgm. The latter investigators feel that Folin's method gives high results and they report a value of 2.35 mgm. using a new method.

Creatinine. From 59 determinations for creatinine we have obtained an average of 1.42 mgm. per 100 cc. with values ranging from 1.19 to 1.94 mgm. With the calves the values decrease with age. Holsteins give the highest average and Jerseys the lowest. There is considerable variation in values reported by other investigators.

Sugar. Our work has given an average of 84.1 mgm. of sugar for 58 determinations with values ranging from 43.2 to 142.0 mgm. A rather striking feature of the data is that blood sugar values decrease as the age of the animal increases. The youngest group gave an average of 105.0 mgm. while the group of older cows gave an average of 51.2 mgm. In other work we have always found low blood sugar values with cows which are lactating heavily. Our results for cows agree very closely with those found by Hayden and Scholl (19).

Chlorides. As a result of 55 analyses, we report an average of 492 mgm. of sodium chloride per 100 cc. The results range from 426 to 546 mgm. The animals in the group less than 1 month of age gave an average of 469 mgm. which is the group showing the greatest variation from the total average which we report. The value of 532 mgm. for Brown Swiss-Jersey is the result for one animal. Our average is about an average of results reported by other investigators.

Inorganic phosphorus. As a result of 20 analyses, we obtained an average of 4.46 mgm. of inorganic phosphorus per 100 cc. of serum, with values ranging from 3.09 to 6.17 mgm. The conspicuous thing about these results is that as the age increases the phosphorus values decrease. The group of youngest calves gives an average of 5.06 mgm., while the cows give a value of 3.62 mgm. per 100 cc. of serum. Females give a higher average than males and Ayrshires and Guernseys give higher averages than Holsteins and Jerseys. There is considerable variation in the values reported by other investigators.

Inorganic plus hydrolyzable organic phosphorus. In the work reported on blood phosphorus, we have found that in most cases only inorganic phosphorus has been considered. Meigs, Blatherwick and Cary (22) have reported on phosphorus distribution in blood plasma, but the distribution is not analogous to that which we report.

As a result of 16 determinations of inorganic plus hydrolyzable organic phosphorus in blood serum, we have found an average of 6.12 mgm. per 100 cc. with values ranging from 3.46 to 7.9 mgm. Here, again, the very young animals showed higher values than the more mature ones. The average for females is nearly twice that for males. By deducting the average inorganic phosphorus from the average of the combined forms, we get 1.17 mgm. for the average hydrolyzable organic phosphorus in 100 cc. of blood serum.

Total acid soluble phosphorus. Hayden and Fish (18) report an average of 9.29 mgm. of acid soluble phosphorus per 100 cc. of serum as a result of 29 determinations on cows.

From 14 determinations we report an average of 7.46 mgm. of total acid soluble phosphorus per 100 cc. of serum, with values ranging from 5.30 to 8.57 mgm. Our results show a considerably higher average for calves than for older animals. Our results on cows are much lower than those reported by Hayden and Fish (18).

From all our results on the various forms of phosphorus in blood serum, it is clear that the serum of young growing animals has a higher phosphorus content than that of mature animals. This is to be expected when one considers the phosphorus requirement for bone-building in young animals. The lower phosphorus content of the serum of mature cows is also reasonable when one considers the large amounts of this element which are secreted into the milk.

Calcium. The results which we report for calcium gave an average of 12.63 mgm. per 100 cc. of serum for 55 analyses, with a range of 9.96 to 16.18 mgm. The results indicate no noticeable differences in the calcium content of young and mature animals. The average for 5 cows from four to nine years of age is 13.31 mgm., while that for 9 calves ranging from one to twenty-eight days of age is 13.22 mgm. Our results are quite definitely higher than those of all the other investigators except Hart and his co-workers (16).

Carbon dioxide. In our work we have determined carbon dioxide on 36 individuals with results ranging from 31.5 to 76.8 cc. per 100 cc. of plasma. The average value is 58.93 cc. A noticeable observation is that the plasma of cows' blood binds less carbon dioxide than that of calves. This has also been noted by Blatherwick (6). Males give a higher average than females. Our average value is very close to that found by Robinson and Huffman (23).

SUMMARY

Fifty-nine samples of blood from dairy calves and cows have been analyzed for hemoglobin, non-protein nitrogen, urea nitrogen, uric acid, creatine, creatinine, sugar, chlorides, phosphorus, calcium and carbon dioxide binding capacity. The results are given in tables 1 and 2.

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(24) Tisdall, F. F.: A note on the Kramer-Tisdall method for the determination of calcium in small amounts of serum. Jour. Biol. Chem., lvi, 439–441, (1923).

(25) Van Slyke, D. D.: Studies of acidosis. A method for the determination of CO_2 and carbonates in solution. Jour. Biol. Chem., xxx, 347–368, (1917).

(26) Van Slyke, D. D.: Gasometric determination of the oxygen and hemoglobin of blood. Jour. Biol. Chem., xxxiii, 127–132, (1918).

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(28) Whitehorn, J. C.: A system of blood analysis. Supplement II. Simplified method for the determination of chlorides in blood or plasma. Jour. Biol. Chem., xlv, 449–460, (1921).

THE PRODUCTION OF "SLIPPED TENDON" IN CHICKS BY EXPERIMENTAL FEEDING*

J. E. Hunter *and* E. M. Funk
The Pennsylvania State College

MANY poultrymen are confronted with a leg deformity in young growing chicks commonly spoken of as "slipped tendon" or "hock disease". The condition is usually associated with brooding on wire, lack of exercise, or crowded conditions.

This abnormality commonly appears in chicks from 3 to 6 weeks of age, but has been noted to appear in birds as old as 8 weeks. An examination of an afflicted bird reveals an enlargement of the hock joint and it is noted that the gastrocnemius tendon slips from its normal position new position at the side of the joint. A in the groove back of the leg and takes a dissection study confirms these observations and further shows that after the tendon has slipped, it proliferates around the joint and strengthens it, but leaves the bird in a deformed condition. The accompanying photograph, showing cross sections of these joints, reveals the abnormal position of the tendon.

One or both legs of the bird may be affected. In order to show that the deform-

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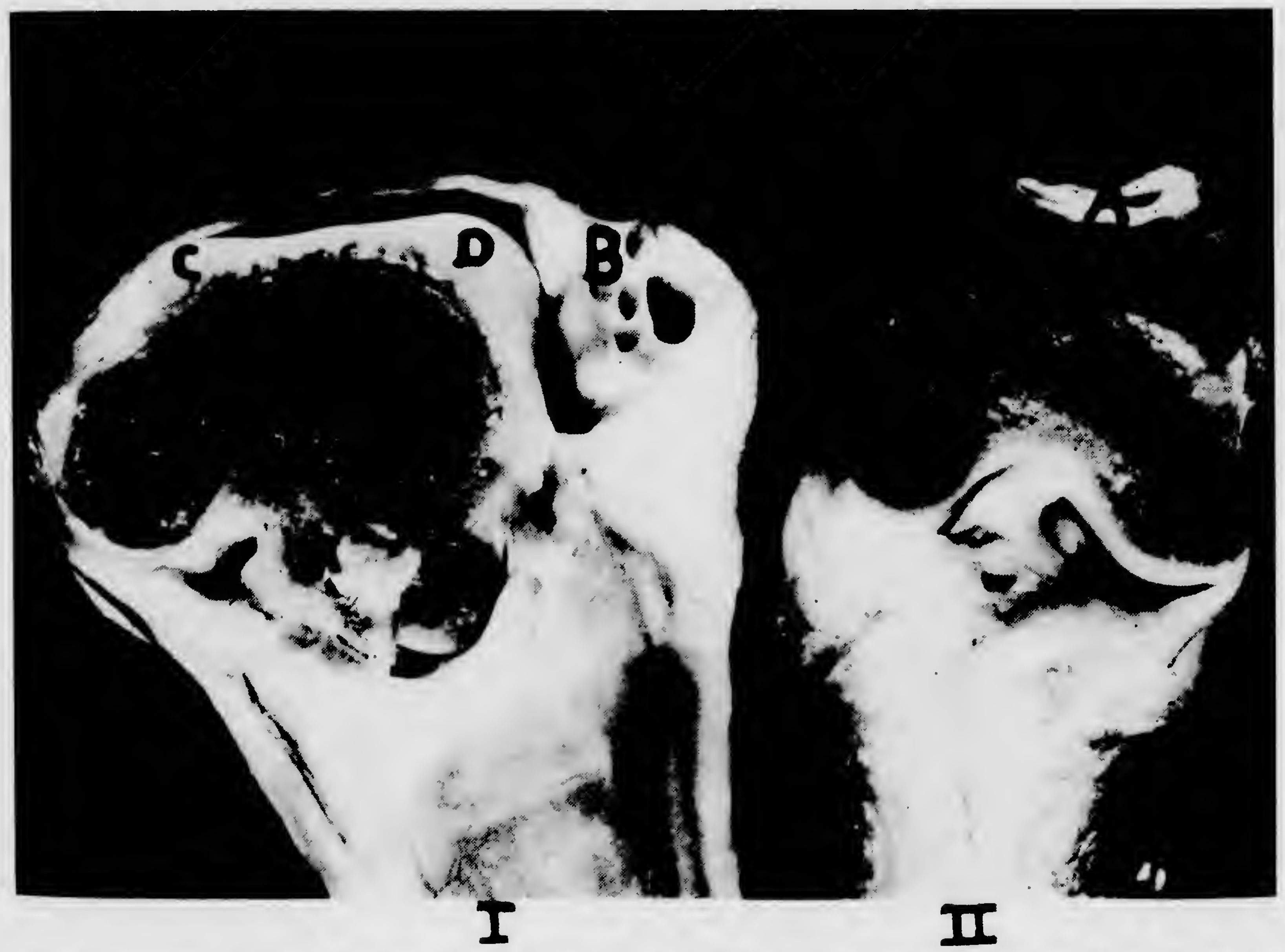

Cross sections of hock joints of a Single Comb White Leghorn chick. — Age—8 weeks.
I. Joint showing "slipped tendon" condition.
B. Tendon slipped from condiles.—Note proliferation around joint.
C. and D. Condiles.
II. Normal joint.
A. Tendon occupying its normal position.

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INTENTIONAL 2ND EXPOSURE

THE PRODUCTION OF "SLIPPED TENDON" IN CHICKS BY EXPERIMENTAL FEEDING*

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The Pennsylvania State College

MANY poultrymen are confronted with a leg deformity in young growing chicks commonly spoken of as "slipped tendon" or "hock disease". The condition is usually associated with brooding on wire, lack of exercise, or crowded conditions.

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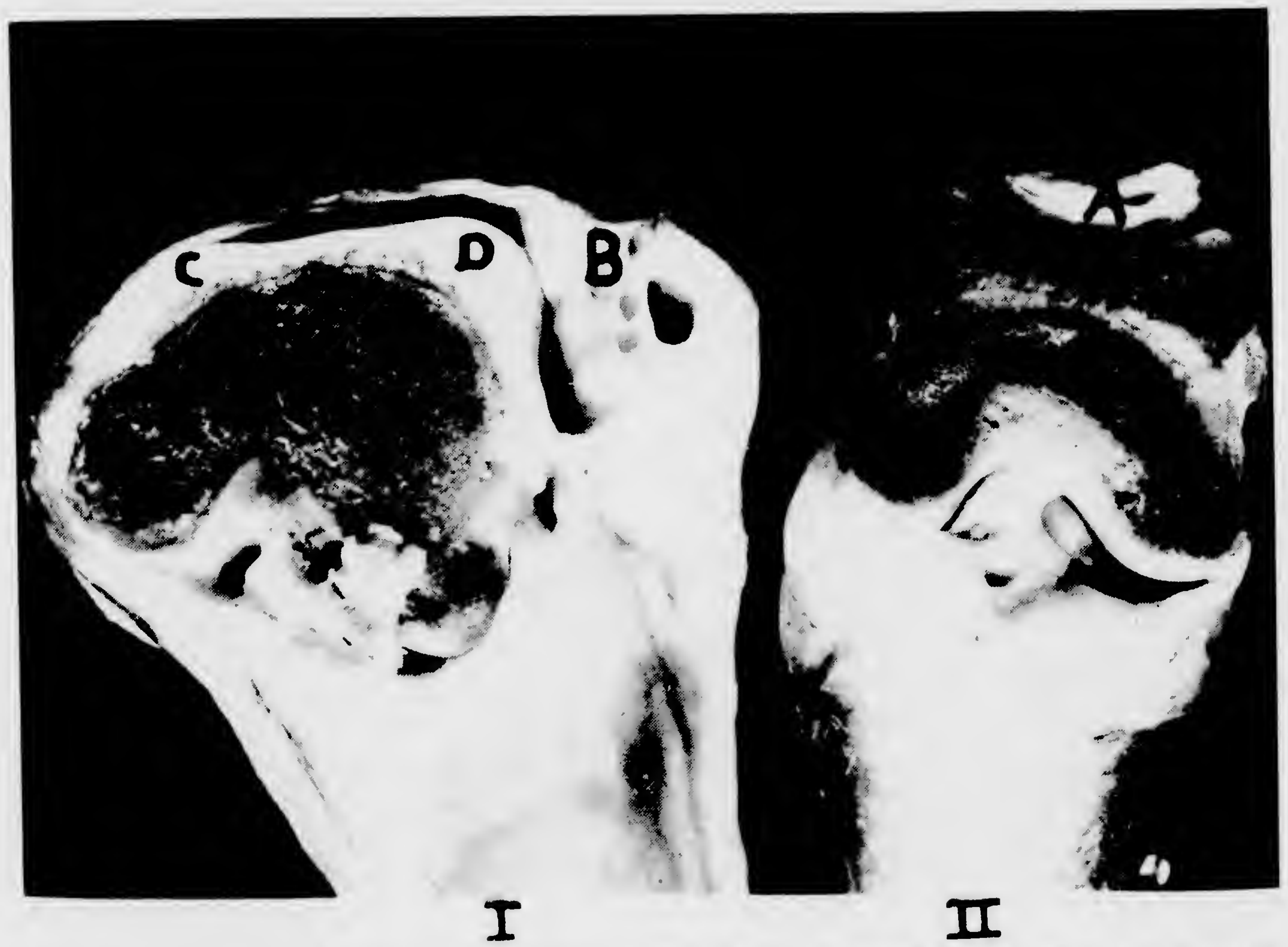
One or both legs of the bird may be affected. In order to show that the deform-



Cross sections of hock joints of a Single Comb White Leghorn chick. — Age—8 weeks.

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B. Tendon slipped from condiles.—Note proliferation around joint.
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ity is not analogous to rickets, chemical as well as histological studies were made of the tibiae of a number of afflicted birds. The bone ash was found to be normal and a normal calcification was revealed by the line test.

Previous observations had shown that a ration of the following composition, fed to Single Comb White Leghorn chicks, would cause approximately 50 per cent of them to become afflicted with "slipped tendon" if brooding was carried out on wire in battery brooders. These brooders consisted of 6 pens each measuring 36" x 36" and 12" high. Unless otherwise stated the experiments described in the rest of this paper were carried out with the birds standing on one-half inch mesh wire screens. When solid floors are specified, it means that a solid platform, covered with sand, replaced the screen in the bottom of the pen. The ration was fed as an all mash ration and was kept before the birds at all times. Day old Single Comb White Leghorns were used in all trials. Composition of the basal ration used in the following trials:

Yellow corn	70.0
Alfalfa leaf meal	3.0
Wheat middlings	5.0
Dried buttermilk	15.0
Meat and bone meal	2.5
Fish meal	2.5
Steamed bone meal	2.0
Cod liver oil	1.0

When this ration was fed to birds brooded on solid floors there were no cases of "slipped tendon" noted. This observation seems to indicate that the ration is about on the borderline, for it is bad enough to produce "slipped tendon" on wire but not bad enough to cause it in chicks brooded on floors. It was found that birds fed the basal ration but raised for the first four weeks of their lives on a solid floor never became afflicted with this abnormality, even though they were transferred to wire floors at the end of this period, and kept there throughout the duration of the experiment. Which fact seems to indicate that the mechanism involved in the production of "slipped tendon" manifests itself early in the life of the chick. So far as "slipped tendon" is concerned, the first few weeks of the chick's life seems to be the critical period in skeletal and tendon development.

When the basal ration was supplemented with 1.5 per cent of sodium phosphate (NaH_2PO_4) it was found that the incidence of "slipped tendon" was greatly increased. Since the addition of phosphorous upsets the Ca:P ratio in the feed, another group was given the basal ration and in addition was given 1.5 per cent of McCollum's salt mixture in place of the sodium phosphate. This salt mixture has the following composition and is supposed to contain the elements in the proportion in which they are needed for bone development:

McCollum's salt mixture No. 185.

NaCl	.173
$MgSO_4$ (anhydrous)	.266
NaH_2PO_4 (hygroscopic)	.347
K_2HPO_4	.954
$CaH_4(PO_4)_2H_2O$	.540
Fe citrate	.118
Ca lactate	1.300

When the above mixture was added to the basal ration the occurrence of "slipped tenden" was only slightly lower than in the trial where sodium phosphate was added. These results seem to indicate that this abnormal condition is aggravated by the supply of minerals in the ration. Card[2] has suggested that ordinary commercial meat and bone meal or meat meal commonly contain about as much "bone meal" as the chick can tolerate, provided the protein is fed at levels above 15 per cent.

In the following feeding trials, the mineral level in the ration was changed by changing the level of bone meal, or meat and bone meal, or fish meal. The fish meal used in the ration had an ash content of 26.2 per cent and the meat and bone meal contained 25.9 per cent ash. The protein level was kept constant by replacing any deductions of protein with commercial casein and when additional fish meal or meat and bone meal were added to the ration enough dried buttermilk was removed to prevent a change in the protein level. Mineral additions to the ration were made at the expense of the corn. When the bone meal was taken from the ration nothing was substituted in its stead and consequently, in such cases, a slight variation occurs in the percentage composition of the ration. The following table shows the results of sixteen feeding trials in summarized form.

TABLE I

Showing Changes in the Ration and the Occurrence of "Slipped Tendon"

Ration	Time of observation Weeks	Floor or wire screen	Number of chicks in group	Number of birds afflicted	% of "Slipped tendon" in group
Basal	8	wire	22	11	50.0
Basal	8	floor	28	0	0
Basal	8	(Wire 4 wks.) (Floor 4 wks.)	28	0	0
Basal	6	wire	22	10	45.5
Basal	6	floor	24	0	0
Basal + 1.5% NaH_2PO_4	8	wire	24	23	95.8
Basal + 1.5% McCollum's salt mixture	8	wire	27	22	81.5
Basal + 1.5% NaH_2PO_4	6	wire	22	20	90.9
Basal + 1.5% NaH_2PO_4	6	floor	23	14	60.9
Basal — fish meal	6	wire	21	6	28.6
Basal — meat and bone meal	6	wire	24	6	25.0
Basal — fish meal — meat and bone meal	6	wire	23	4	17.4
Basal — bone meal	6	wire	25	0	0
Basal + 2% bone meal + 1% NaCl	6	wire	22	18	81.8
Basal + 2½% fish meal + 2½% meat and bone meal	6	wire	23	17	78.9
Basal — fish meal — meat and bone meal — bone meal	6	wire	21	0	0

It will be noted from Table I that when the basal ration is supplemented with additional minerals "slipped tendon" occurs on solid floors as well as on wire but to a lesser extent. When additional minerals in the form of bone meal, sodium phosphate, or McCollum's salt mixture are added to the ration, or when the fish meal or meat and bone meal of the ration are increased, thereby raising the mineral level, it is noted that more birds become afflicted with this abnormality than when they are fed the unsupplemented basal ration. It is also observed that when minerals are taken from the ration by removing the meat and bone meal or fish meal or both, the occurrence of "slipped tendon" is lessened. When the bone meal is omitted from the ration no cases of the deformity appeared. Payne[3] describes the condition of "slipped tendon" and points out that the trouble has not been confined to any group of conditions and that it occurs with rations having low and high mineral contents. It will be noted that the basal ration used in the described trials is not rich in minerals but that the addition of minerals in several different forms greatly increases the production of "slipped tendon". The ration is very low in fiber and this may be a factor in the production of this abnormality. The skeletal system of a chick brooded on wire seems to undergo a strain but this strain is lessened by brooding on solid floors. It is very probable that the brooding of chicks on wire discourages activity and causes the bird to take less exercise than when brooding is carried out on floors.

From the above data it seems apparent that the mineral content of the ration is an etiological factor in the production of "slipped tendon". There are of course other contributing factors of which probably the most important is the lack of exercise.

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[1] McCollum, E. V., J. Biol. Chem. XXXII, 1917, 181.

[2] Card, L. E., A Year's Progress in Solving Farm Problems in Illinois, 1928-29, 114.

[3] Payne, Loyal F., Science, LXXI, No. 1852, 1930.

Reprinted from Science, October 24, 1930, Vol. LXXII,
No. 1869, pages 425–427.

THE CONCEPTION OF BALANCE WITH RESPECT TO THE ABSORPTION OF NITROGEN, PHOSPHORUS AND POTASSIUM BY PLANTS AND THE INFLUENCE OF THE LEVEL OF NUTRITION

In a recent paper, the writer[1] called attention to the remarkable and consistent results obtained over a 10-year period by Lagatu and Maume[2] from a series of field experiments conducted with the grape (*Vitis vinifera*). These authorities concluded that the depression in yields produced by the application of an "incomplete" fertilizer, *i.e.,* one containing only two of the principal fertilizer constituents, nitrogen, phosphorus, potassium, is not due to a *depression* of the absorption of the remaining elements, but, on the contrary, to a nutritional lack of balance owing to an *increased* absorption of these elements.

Field experiments have also been reported[3] in which it was noted that the omission of potassium from a fertilizer applied to a soil deficient in this element resulted in an *increased* absorption by the plants of the nitrogen and phosphorus present in the "incomplete" fertilizer. But in these experiments of Wallace it is to be noted that the omission of nitrogen from the fertilizer resulted in a *decreased* absorption of phosphorus and potassium, and the omission of phosphorus in a *decreased* absorption of nitrogen and potassium.

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[1] Walter Thomas, Science, 70: 382–384, 1929.

[2] H. Lagatu, *Compt. Rend.*, 172: 129, 1921; H. Lagatu and L. Maume, *Compt. Rend.*, 179: 782, 1924; *ibid.*, 179: 932, 1924; *ibid.*, 180: 1179, 1925; ''Communication au Congrès des engrais azotés de synthèse à Montpellier,'' Juin 1, 1927, pp. 1–15.

[3] T. Wallace, *Jour. Pomol. and Hort. Sci.*, 7: 130–145, 1928.

In the course of experiments[4] with *Pyrus malus* L. grown under controlled conditions, the writer has had a unique opportunity of examining the principles enunciated by Lagatu and Maume. It has been found in our experiments that the course of absorption of nitrogen, phosphorus and potassium from fertilizer mixtures containing only two of them is in accordance with the deductions made by Liebig.[1] Thus, a comparison of the absorption graphs of trees receiving additions of two only of the elements, nitrogen, phosphorus, potassium, with the absorption graphs of trees which received additions of all these elements indicates a depression of the absorption of the elements from the "incomplete" or unbalanced fertilizer.

Since, therefore, generalization of Lagatu and Maume's principles is not permissible, it is pertinent to seek an explanation of the causes operative. The discrepancy between the results of the experiments under discussion may not be attributed to differences in the ratios, amounts or composition of the fertilizers applied, for these are very similar. The conception of physiological balance resulting from Loeb's[5] pioneer experiments and its further expansion by McCool,[6] Osterhout[7] and others have stimulated a vast amount of investigation by plant and animal physiologists and, although quantitative experiments[8] with plants have been made to ascertain the factors influencing the selective absorption (diffusion) into the cell of one salt (or ion) by another present in the nutrient solution, the discovery of a general law applicable under all conditions has not been forthcoming. It is, however, apparent from such experiments that there exists for each species a physiologically balanced nutrient solution—which may in actual field practice be determined by the method of Mitscherlich[9]—from which normal permeability occurs; and that a departure from this balance will produce a disturbance in the rate of absorption relations of the various ions that may have a profound effect on metabolism.[7] Normal permeability has been explained[10] on the basis of antagonistic salt action; but the extent to which salt (or ion) antagonism is responsible for the maintenance of normal permeability of plants grown under field conditions is problematical. In the field experiments here cited the results do not appear to be applicable on the basis of simple antagonism—at least in the sense defined by Loeb[10] and as discussed more recently by Loehwing[11]—for plants grown on these soils containing normal concentrations of calcium ions absorbed more (not less) potassium from a fertilizer containing only nitrogen and phosphorus. There is evidence,[7] moreover, to show that antagonism becomes weaker and weaker as the concentration decreases. Thus, Osterhout found that although 0.05 M $NaCl + 0.06$ M $CaCl_2$ exerted a marked toxic effect on root growth, 0.001 M + 0.0012 M solutions, respectively, of this mixture had no antagonistic effect. We should expect, therefore, little or no antagonistic effect in solutions of such low concentration as that of the soil solution. For example, the concentration of salts in the soil in the present experiments varied from 350 to 640 p.p.m. according to the season. In all nutrient culture experiments with seedling plants in which the phenomenon of antagonism has been observed, the concentration of salts present is from five to twenty times that of the salts in the soil solution from normal soils.

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[4] Walter Thomas and R. D. Anthony, *Proc. Am. Soc. Hort. Sci.*, 81–87, 1926; Walter Thomas, *Plant Physiology*, 2: 109–137, 1927.

[5] Jacques Loeb, "Opperheimer's Handbuch der Biochemie des Menschen und der Tiere," Zweiter Bd., Teil I, Gustav Fischer, Jena, 1909.

[6] M. M. McCool, Cornell Univ. Agr. Exp. Sta. Memoir No. 2, 119–216, 1913.

[7] W. J. V. Osterhout, "Injury, Recovery and Death in Relation to Conductivity and Permeability," J. B. Lippincott Company, Philadelphia, 1922.

[8] D. D. Waynick, *Univ. Calif. Pub. Agr. Sci.*, 3: 135–242, 1918; D. R. Hoagland and J. C. Martin, Univ. Calif. Pub. Agr. Exp. Sta., Tech. Paper No. 8: 1–26, 1923; H. S. Reed and A. R. C. Haas, *Jour. Agr. Res.*, 24: 801–814, 1923; Univ. Calif. Pub. Agr. Exp. Sta., Tech. Paper No. 11: 1–23, 1923; Univ. Calif. Pub. Agr. Exp. Sta., Tech. Paper No. 17: 1–75, 1924; D. R. Hoagland and A. R. Davis, *New Phytologist*, 24: 99–111, 1925; E. Pantanelli, *Protoplasma*, 7: 129–137, 1929.

[9] A. Mitscherlich, "Die Bestimmung des Düngerbedürfnisses des Bodens," Paul Parey, Berlin, 1925.

[10] Jacques Loeb, "Dynamics of Living Matter," Columbia University Press, New York, 1906; *Biochem. Ztschr.*, 32: 308–322, 1911; SCIENCE, 36: 637–639, 1912.

[11] Walter F. Loehwing, *Plant Physiology*, 3: 261–275, 1928.

However, although ion antagonism may be of negligible consequence in solutions as dilute as the soil solution, experimental evidence exists to show that the concentration of nutrients (level of nutrition,[9, 12] *i.e.,* rate of supply of nutrients[13] or supplying power[14]) of the soil solution may be the factor of greatest influence in determining the course of absorption. Thus, from Remy's[15] numerous field experiments it is apparent that the addition of any two of the elements, nitrogen, phosphorus, potassium, to soils relatively deficient in these elements, such as the Hagerstown clay loam soil used in the experiments of the writer, results in a *decrease* in the absorption of the remaining element in accordance with the deductions of Liebig.[1] This decreased absorption does not occur on soils—such as that used by Lagatu and Maume[2]—well supplied with these elements, but always an increased absorption.[15, 16] In this connection it is of interest to note that Waynick[17] observed in culture solutions greater antagonism between 0.04 M $MgSO_4 + 0.18$ M KCl than from mixtures containing higher concentrations of $MgSO_4$.

The factors involved in the absorption of salts (or ions) by plants have been discussed by the writer,[18] and a recent paper by Cooper[12] presents some stimulating new ideas on the subject. The causal relations are known to be extremely complex. Nevertheless, although the factors producing differential absorption and influencing utilization of elements within the plant at the different planes of nutrition may not at the present stage of our knowledge be identified, the interpretation advanced to account for the discrepancy between Lagatu and Maume's results and those of the writer is the only one that, at the present stage, accounts for the observed facts. Details of the experiments will be published elsewhere.

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[12] H. P. Cooper, *Plant Physiology,* 5: 193–214, 1930.

[13] D. R. Hoagland, *Jour. Agr. Res.,* 18: 73–117, 1919.

[14] Burton E. Livingston, Proc. Intern. Congress Plant Sciences, Ithaca (1926), Vol. 2, pp. 1107–1121, the Collegiate Press, Menasha, Wisconsin, 1929.

[15] Theodor Remy, ''Untersuchungen über das Kalidüngerbedürfnis der Gerste,'' Paul Parey, Berlin, 1898.

[16] F. Sekera, *Ztschr. für Pflanzenernähr. u. Düngung,* 7–B: 533–539, 1928.

[17] D. D. Waynick, *Univ. Calif. Pub. Agr. Sci.,* 3: 135–242, 1918.

[18] Walter Thomas, *Soil Sci.,* 27: 249–270, 1929; *Plant Physiology,* Vol. 5, No. 4, 1930 (forthcoming).

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THE FEEDING POWER OF PLANTS*

WALTER THOMAS

A. Introductory

1. VARIABLE COMPOSITION OF IDENTICAL SPECIES

The publication of WOLFF'S monumental work on "Aschen-analysen" in 1871 (**210**), although compiled principally for the purpose of supplying information on the composition of plants used in feeding farm animals, nevertheless, served to crystallize the large amount of experimental data of English, French and German investigators that had accumulated even in those early days of agricultural science. WOLFF'S data showed very clearly that plants even of the *same species* differed considerably both in the *absolute* and *percentage amounts* of the various elements absorbed, depending upon the composition and nature of the substrate (soil or nutrient solution). Indeed, WOLFF himself (**209**) was the first to give definite proof that the composition of the ash of any plant could be changed by varying the proportion of salts in the nutrient culture medium.

2. SELECTIVE POWER OF DIFFERENT SPECIES

Numerous investigations, those of NEWTON (**129**) being the most recent, have since shown, moreover, that *different species* growing in media of identical composition possess selective powers with respect to any specific ion or ions. This marked difference in the assimilatory powers of different plants is very clearly exhibited in their different responses to such "insoluble" mineral fertilizers like rock phosphate, basalt, gneiss, etc.

3. ARE DIFFERENCES IN RESPONSE A SPECIES CHARACTERISTIC

But as the environmental conditions even in the water and sand culture experiments cited by WOLFF were never identical, the question as to the nature of the factors operative was still left open. Nor was it decided whether the differences in responses exhibited were characteristic of the species or the result of environmental or other unknown factors. And, although Newton (**126**) believes that with the exception of phosphorus (**129**) there is no characteristic difference in the *type of absorption* of certain species of plants (barley and peas, for example) in nutrient culture solutions, *i.e.*, that there is no difference in percentage content with respect to any element in the plants examined that holds consistently in favor of one or the other throughout all stages of growth, it will be apparent as the subject is developed that the evidence is overwhelming that different plant species growing in media of identical composition not only exhibit differences in composition that can only be interpreted to indicate marked differences in the type of absorption (**5**) but that they exhibit also differences in what has been frequently designated in the literature by the expression feeding power or ability to utilize insoluble soil constituents to different degrees, which are characteristic of the species. The following data from König's experiments (**82**)—which are among the earliest—are typical as exemplifying this point:

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* Publication authorized by the Director of the Pennsylvania Agricultural Experiment Station as technical paper no. 502.

TABLE I

Per cent. of K_2O in oats and peas grown under similar conditions

		Sandy	Loam	Loam	Calcareous	Clay
Yields (first year)	Oats	0.396	0.506	0.420	0.489	0.401
	Peas	0.554	0.688	0.554	0.666	0.702

4. The term ‘‘feeding power’’ defined

The term ‘‘absorbing power’’ or ‘‘feeding power’’ as it is known in technical agronomy may connote several ideas (**67**). Many have objected to the use of the term feeding power. Shull (**172**) suggests that some plant physiologists prefer not to use the term on the basis that it has too much psychological implication and others (**67**) because of the many different interpretations given to it. But, as Crocker (**27**) points out, although the expression has undesirable implications, it is better to continue its use than to invent some new phrase that might, perhaps, be freer from these implications, because other terms suggested, like ‘‘absorbing power of crop plants’’ or even ‘‘specific absorbing power,’’ do not imply quite enough. The term ‘‘feeding power’’ implies both power to absorb the soil nutrients and also the power of making use of many of the salts absorbed. This expression will be used in the present paper as a concise means of indicating the difference exhibited by different types of plants in the *absorption* and *utilization* of difficultly available minerals, either native to the soil or added as soil amendments, as determined by the differences in the amount of nutrient elements absorbed from the minerals in question, the utilization of which is generally exhibited in different growth responses.

5. Theories advanced to account for different responses

From the numerous experiments conducted to determine the cause of the characteristic differences in absorbing power by different species, a number of explanations have been advanced. The differences have been attributed to (1) the differences in the chemical nature (and possibly amount also) of other products of root exudations (acids, carbohydrates, etc.); (2) the differences in (a) the absolute amounts of carbon dioxide evolved, or (b) the amounts evolved per gram of dry weight of the roots (respiration energy); (3) the operation of the law of mass action between the carbon dioxide (carbonic acid) from the roots and the ‘‘insoluble material’’ in the soil; (4) unlike potential differences between the soil and plant; (5) differences in the permeability of the root membranes producing ‘‘selectivity’’ in absorption of roots; (6) the existence of a Donnan membrane equilibrium, and to a combination of one or more of the above factors (**67**).

We shall discuss the evidence for the operation for these various factors separately.

B. The chemical nature of the root exudations

1. The carbon dioxide factor

The problem of determining the nature of root secretion is of importance not only from the standpoint of scientific inquiry, involving the fundamental question of the mechanism of absorption of nutrients and the reaction of protoplasm, but is of importance also from the practical aspect with respect to the economic utilization of minerals occurring naturally in the soil, and applied also as soil amendments.

a. Considerations of some of the experimental difficulties involved.—It must be granted that even if proof were forthcoming that carbon dioxide is the only factor involved, as indeed Hall (**55**) points out, the problem of isolating the factors that contribute to the sum total of the soil carbon dioxide is not a simple one. In the first place, the carbon dioxide is not all in the gaseous phase, a large part (**163**) (and, indeed, under some conditions nearly all) is present in solution (**93**); and in the second place, the soil respiration is the total of all the soil processes that give rise to the production of CO_2, *viz.*, oxidation (**83, 169, 213**), the metabolic processes of the microorganisms (**110, 181, 182, 183, 213**), and the respiration of roots and of the bacteria that inhabit them (**58, 63, 110, 178, 181, 208**). It follows that the concentration of carbon dioxide, as measured in most experiments conducted with soils, is a function of the absolute CO_2 produced by the soil particles together with the roots, and also of the diffusion velocity (**102, 103**).

Since the distribution of the CO_2 between the gaseous and liquid phases will depend upon a number of variables (water and calcium principally), the net effect of any one factor at any time is necessarily difficult to determine. Russell (**162**) believes that the CO_2 evolved from plant roots cannot be distinguished from that given off by microorganisms and this is also the conclusion drawn by Starkey (**177, 178**); but the work of Stoklasa (**182**), Headden (**60**), Turpin (**198**), Barakov (**3**) and Neller (**124**) indicates that approximate relative differentiation is possible.

b. The views of the several schools.—Plant physiologists and soil scientists have been and still are divided into several schools. One group (**10, 155, 162**) maintains that plants do not dissolve *significant* quantities of minerals that would otherwise remain undissolved; another school emphasizes the important rôle that the root exudations exercise in this connection. The latter school is divided into two groups: One (**26, 38, 39, 49, 77, 89, 96, 141, 145**) subscribing to the view that "insoluble" substances are rendered soluble by the exudation from the roots of plants of substances (*e.g.*, organic acids, acid salts, etc.) other than carbon dioxide; and the other school (**1, 52, 55, 60, 117, 121, 182**) that only CO_2 is exuded from the roots and that the influence of plants on the solubility of soil minerals is to be attributed solely to this factor. The rôle played by microorganisms in the soil and especially the action of microorganisms of the rhizosphere, isolated by Stoklasa (**181**) is recognized by all schools.

c. Characterization of object of experiments in this field.—Consequently, in the examination of the available evidence for the purpose of evaluating the contribution of the carbon dioxide evolved from plant roots as a factor in dissolving the "insoluble" soil materials, we must distinguish between (1) those experiments (**3, 31, 59, 60, 84, 85, 86, 92, 102, 103, 163, 182, 198, 211, 212**) that have for their purpose the determination of the relative contribution of carbon dioxide by root and microorganisms of the soil, and (2) those (**28, 38, 49, 77, 89, 117, 121, 145**) which, although accepting the important rôle of the carbon dioxide evolved from the roots, were carried out for the purpose of determining if acids and other substances than CO_2 are exuded by roots, and (3) those (**37, 139, 140, 181, 191**) that are concerned with the effect of CO_2 from whatever source derived.

d. Observations of the earlier investigators.—The older "corrosion" experiments of Sachs (**165**), Knop (**80**), Liebig (**97**), Kny (**81**), Czapek (**28, 29**) and Molisch (**122**) and observations of the natural etchings frequently found upon calcareous stones lying in the soil were regarded as furnishing *prima facie* evidence that the roots of plants participate in dissolving "insoluble" nutrients of the soil. And more recently Fred and Haas (**44**) found that this action of roots on marble is increased when a sterilized soil was inoculated with pure cultures of various bacteria.

The growth fusion ideas (**26, 176**) and etching experiments of Sachs (**165**) and of Czapek (**29**) are of interest here. The former observed that only the smooth surfaces of the minerals that were soluble in carbonic acid showed "corrosion figures"; silicates did not. Czapek (**29**) sought further information on the problem by substituting plaster of Paris slabs which were incorporated with various carbonates and phosphates. He argued (**28, 29**) that since—in addition to the carbonates—the phosphates of calcium, magnesium and iron, but not of aluminum phosphate, were corroded by roots, the acids exuded from the roots must be limited to carbonic, acetic, propionic and butyric. Finally, all but carbonic acid were eliminated by means of qualitative results with Congo red. Czapek's deduction from these experiments, however, is not valid, for the evidence from the experiments of Cameron and Hurst (**20**), Prianishnikov (**151, 153, 155**) and Marias (**107**) is conclusive that aluminum phosphate is available to plants through hydrolytic processes. The use, therefore, of $AlPO_4$ is not justifiable in experiments to decide the nature of root exudations.

Moreover, all these "corrosion" experiments may be criticized on the basis that no mineral phosphates exist that are unavailable to plants when all factors that hinder the dissolving power of the roots are removed. The experiments of Butkewitsch (**16**), which will be discussed later in this paper, exemplify this very clearly.

e. The use of seedlings in determining the nature of root exudations.—The approach to the problem by the use of seedling plants, moreover, is not sound (**1, 28, 29, 52**). The mineral requirements of such young plants are too small to justify the suitability of such experiments to answer the question of root exudations, by whatever method and however carefully carried out. The conclusions from such experiments must be of doubtful value. For this reason we shall consider only those experiments in which mature plants were used.

f. The problem of interpretation.—The results of field plot experiments (**59, 60, 92, 102, 103, 163, 211**) are contradictory, but pot and lysimeter experiments (**3, 92, 128, 182, 198**), with certain exceptions (**86**) where the technique adopted may be open to criticism (**55**), agree in attributing to the roots a considerable and, indeed, preponderant contribution to the carbon dioxide of the soil.

(1) The work of the Rothamsted group.—Notwithstanding the fact that on the Broadbalk field and Hoos plots Russell and Appleyard (**163, 164**) found, in agreement with other investigators (**3, 9, 59, 60, 92, 198**), that at certain seasons of the year, especially at the time of active growth and ripening, cropped soils contain a higher content of CO_2 than uncropped soils, their interpretation of the results differ. Russell and Appleyard (**163**) attribute the conflicting results of the older workers (**40, 41, 123, 211**), even when comparison was made between cropped and fallow portions of the same plot, to failure to recognize the marked influence of differences in the physical and chemical structure on the carbon dioxide content of the soils in question. The Rothamsted investigators conclude that in their own experiments the difference in the physical structure and composition of the cropped (or grass) and uncropped (fallow land) soils makes comparison difficult. These authorities observed that the smaller the soil differences become, the smaller is the effect of the crop on the production of CO_2 and they postulate that, if it were possible to obtain absolute identity of conditions, the effect of the crop would vanish. But that the factor assigned by these authorities for the observed differences cannot be the only one is shown by the work of Headden (**60**), who found a marked depression of soil carbon dioxide after each cutting of alfalfa. There is, moreover, such a marked difference in the amount of CO_2 evolved from an uncropped and a cropped plot in some of the more recent field experiments (**59**) that it is difficult to conceive how such marked divergence could be due to physical differences in the soil of the plots. For example, Hasse and Kirchmeyer's (**59**) results indicate that four-fifths of the total CO_2 was due to root respiration.

(2) Are lysimeter results applicable to field conditions?—Moreover, if Russell and Appleyard's (**163**) conclusions are generally applicable, the question may be raised whether pot and lysimeter experiments of the type conducted by Barakov (**3**), Turpin (**198**) and others (**9, 37**) are applicable to field conditions. That the deductions drawn from some of these experiments may not be applicable to field conditions, on account of the conditions being too artificial, is possible. This criticism would apply to the conclusions of Kossowitsch (**84, 85, 86**) in which CO_2 was determined in percolates of the nutrient solution in quartz sand cultures, but for the fact that Dustman (**37**) has shown that, although the absolute respiration of plants grown in quartz and soil cultures is greater in the former, the relative differences between different plants are the same in both.

In Turpin's lysimeter experiments (**198**) the difference between the amount of carbon dioxide in the cropped soil and that in the uncropped soil at the *period of most active crop growth*, divided by the amount of water transpired by the crop, gave a constant which varied with the season. Moreover, as there was no indication that the increased CO_2 in the cropped soil arose from the decomposition of root particles, Turpin logically concludes from these experiments that the plant often produces at the period of its most active growth many times as much CO_2 as is produced by soil organisms.

The results of Barakov's lysimeter experiments (**3**), in which weekly determinations of CO_2 were made on different types of unsterilized soils, also indicate that the principal source of CO_2 is from the respiration of roots. The greatest absolute quantity of CO_2 was associated with the greatest development of the plant. He notes that each plant experimented with had its own specific respiration curve.

Starkey (**177, 178**), however, frequently found little effect on the CO_2 content of the soil or abundance of organisms, especially in the early stages of growth. He also noted the occurrence of larger numbers of microorganisms about plant roots than at a distance from them. On the basis of these findings Starkey questions the conclusions of Turpin (**198**) and Barakov (**3**). There is no reason to doubt that at certain periods a part of the carbon dioxide evolved in the respiration of roots is contributed by the bacteria that inhabit the root surface, *i.e.*, the rhizosphere (**58, 110, 180**); but the weight of evidence (**3, 60, 198**) indicates that the total contribution to the soil CO_2 by such means is relatively insignificant compared with the amount evolved during the growing season by the respiratory activity of the roots. It is also apparent on physico-chemical grounds that determinations of carbon dioxide on samples of soil taken under such conditions as those described by Starkey (**177**) afford no basis for drawing conclusions concerning the quantitative relations relative to the carbon dioxide of the soil atmosphere *in situ*. This same disadvantage applies also to Metzger's (**111, 112**) attempt to measure the concentration of bicarbonates in soil samples immediately around the roots with those taken away from the roots, for these methods obviously destroy the CO_2 equilibrium conditions actually existing in the soil. A review of methods used for the determination of carbon dioxide of the soil atmosphere will be found in a paper by Potter and Snyder (**150**)

Hutchinson (**72**) concludes that there exists a parallelism between bacterial numbers and CO_2 content of the soil. This report (**72**) is frequently cited in the literature; but it is sketchy and lacking in details. If the exact parallelism between soil CO_2 and bacterial numbers occurs throughout the growing season, this fact would tend to show that the root contribution would be small; but when such correlations are attempted

(**72, 163**) there are well defined periods when the bacterial numbers fall as the CO_2 increases. If, then, as WOLLNY and others (**144, 198, 213**) suggest, relatively large increases of CO_2 depress bacterial activity, no correlation would be expected, which may, as PLUMMER (**149**) suggests, be due to the limitation of the oxygen supply. It should be noted, however, that STOKLAS (**180**) finds that root respiration and the number of bacteria in the rhizosphere paralleled one another.

We must, therefore, conclude that either RUSSELL and APPLEYARD'S (**163**) results were due to conditions not readily duplicated and that the conclusions drawn by them are not of general application, or that deductions from experiments of this type carried out in pots and lysimeters are not applicable to conditions in the field. The assimilation and utilization of nutrients in pot experiments is considerably greater than in field experiments. In the former the roots penetrate the soil more intensively than in the open field. As a result of this better utilization of nutrients, the yields in pot experiments are frequently six to ten times higher than those in the field. Recent suggestions (**32**) to eliminate these differences include mixing the soil with equal parts *by volume* of quartz sand. Finally, it is to be noted that there is no evidence that the discrepancies in the experiments discussed above can be attributed to the relative predominance of root respiratory activities as a result of the relatively low rate of oxidation of organic matter or *vice versa*.

g. INTENSITY OF CO_2 PRODUCTION BY ROOTS OF DIFFERENT SPECIES.—Since the atomic groupings and reciprocal linkages of the roots of different plants are not similar, it could be postulated that the respiration activity of the root systems of plants would be different. The complexity of the problem is appreciated when specific data for the respiratory energy of the roots of different plants are considered. The causes of the wide variations reported by different investigators for identical types of plants must be sought in the different conditions of experimental technique, especially with respect to the difficulty in making a comparison of the influence of the various factors that influence respiration, such as concentration of oxygen, temperature, concentration of carbon dioxide, and nature and concentration of nutrients.

Thus, the average amount of CO_2 evolved in 24 hours from barley grown to maturity was found by STOKLASA and ERNEST (**182, 183**) to be 70 mg. per gm. dry weight of roots, whereas NEWTON (**127**), in plants 35 days old, found only 3.5 mg. of CO_2 per gm. dry weight of roots evolved in 26 hours. Both of the above investigators used sand as the substrate. STOKLASA used sterilized sand and KNOP'S nutrient solution. NEWTON used HOAGLAND'S culture solution. NEWTON has thought that possibly his method of simple diffusion may not have measured all the CO_2 evolved (**130**). But the agreement between DUSTMAN'S (**37**) results and NEWTON'S shows that this is not the reason and, theoretically, no great difference would be expected to result between systems depending on diffusion and air currents, respectively, since the measurements must necessarily have been made when each system was in a state of equilibrium between output and removal of CO_2 (**104**); nor is there any evidence that these large differences in STOKLASA'S and NEWTON'S values can be attributed to any great difference in the age of the plants used. Values obtained by the writer (**186**) for sweet clover in sand cultures by a similar method to that used by PFEIFFER and BLANCK (**145**) ranged from 8–9 mg. per gram. of dry weight of roots evolved in 24 hours, compared with values of 4.5 mg. obtained by NEWTON. DUSTMAN'S (**37**) results are in relative agreement with NEWTON'S. That STOKLASA and ERNEST'S (**182**) values are far higher than is usual under field conditions will be evident from a consideration of the following facts: STOKLASA concludes that the microorganisms in an acre of soil to a depth of 1.5 feet may produce between 65 and 70 pounds of CO_2 a day for 200 days in the year, and that during the growing period the roots of oats or wheat would give off nearly as much to an acre. This would mean a loss from decomposition of organic matter of 13,500 pounds of CO_2 per acre per year, or $0.471 \times 13{,}500 = 6{,}358$ pounds of organic matter. This value is abnormally high. As NEWTON (**130**) points out, a loss of one-half to one ton of organic matter per acre per year is closer to the average loss from normal soils.

It must be admitted (**87**) that calculation of the carbon dioxide production per gram of dry weight of material is not a very refined method of measurement, owing to the variation in the percentage amount of protoplasm in the various plants and parts of plants, the respiratory gas exchange of which depends entirely upon the activity of the living substance. Some tissues, such as wood and cork, consisting for the most part of dead cells, do not respire at all. But, owing to the impracticability of the alternative methods proposed (**87**), the simple method of calculation on the basis of total weight must be used.

Studied from a *relative standpoint*, however, STOKLASA and ERNEST'S results are decidedly valuable. Thus, the specific respiration energies (amount of CO_2 evolved per gram of dry weight) of roots found by them were barley 68, wheat 90, oats 127, rye 118, buckwheat 227. In sterilized sand culture experiments they found that the above values were in the same relative order as the utilization of P_2O_5 and K_2O from basalt and gneiss. As already pointed out, BARAKOV (**3**), too, in experiments with lupin, oats, barley and rye, found that the capacity to absorb Ca from rock phosphate paralleled their values for the specific respiration energy. Stoklasa and Ernest, whose results are in substantial accord with those of Barakov's (**3**), point out that the values found by them for the respiration energies do not, however, afford any indication of the relative yields under conditions where readily assimilable nutrients are supplied. Thus, in Knop's nutrient solution the yield of barley was found to be far greater than that of wheat, rye or oats. This might be attributed to the compensating factor of extensive root development in the case of barley, as Hesse's measurements (**62**) of the root hairs of barley, oats, rye and wheat indicate and which are in harmony with the measurements of Nobbe (**131**).

TABLE II

Size of root hairs on cereal crop plants

	Length	*Diameter*
	mm.	*mm.*
Barley	1.40	0.0102
Oats	0.98	0.0090
Rye	0.86	0.0070
Wheat	0.62	0.0068

If Stoklasa and Ernest's results can be corroborated we could postulate: (1) That when the rate of supply of N, P and K are sufficiently rapid, as in the case of access to an ample supply of easily soluble nutrients, the yield will be proportional to the extent of root system. (2) But if one of these elements, such as phosphorus, is present only in a difficultly soluble form, the yield will be proportional to the respiration energies.

If these conclusions of Stoklasa and Ernest (**182**) and of the other investigators cited (p. 446) can be proved to be of general applicability to our economic plants, we should be forced to accept the views of the school that regards the rôle played by carbon dioxide as the greatest factor determining their feeding power. The need, therefore, for more exact information on the respiration coefficients of the various types of economic plants is apparent.

2. To what extent does CO_2 function as a solvent

a. The imbibitional water of the root hairs is not a saturated solution of carbon dioxide.—Aberson (**1**), on the basis of titration and hydrogen-ion concentration values obtained on *seedling roots,* concluded that the hydrogen-ion concentration of the water of imbibition of the mucilaginous "membranes" of the root hairs in close contact with the soil particles contains a saturated solution of carbon dioxide. Experimental evidence is cited to show that a saturated solution of CO_2 is able to bring into solution the insoluble soil minerals, especially phosphates.

But Aberson's view is not valid for the following reasons: (1) A saturated solution of carbon dioxide has a hydrogen-ion concentration of 1.2×10^{-4}; this exceeds the hydrogen-ion concentration values of any of the root exudations found by Aberson. (2) With any given content of carbon dioxide in the soil atmosphere it is possible to calculate the content of CO_2 and HCO_3 ions in the water of imbibition in contact with it.

$$\text{Since } HA = H^+ + A^- \tag{1}$$

$$\frac{[H^+]\ [A^-]}{[HA]} = K_a \tag{2}$$

$$\therefore\ [H^+]^2 = K_a \cdot [HA] \text{ or } [H^+] = \sqrt{K_a \cdot [HA]} \tag{3}$$

$$\text{and} \qquad [H^+] = \sqrt{K_a \cdot (C - [H^+])} \tag{4}$$

where C is the concentration of the original acid.

Since $[H^+]$ is small, we have

$$[H^+] = \sqrt{K_a \cdot C} \tag{5}$$

$$\text{or } pH = -1/2 \log. K_a + 1/2 \log C \tag{6}$$

For polybasic acids the first ionization constant may be taken, since the second ionization constant is small compared with the first.

Since the Henry coefficient at N.T.P. is approximately 1:1 (**199**), the percentage by volume of carbonic acid of the soil solution and the percentage in the soil air are approximately identical. Hence, even if the soil air contained as high as 10 per cent. CO_2, the soil solution (if it consisted of pure water) would also contain 10 per cent. by volume of CO_2, since the Henry coefficient is 1:1. The pH of such a solution calculated from the above equation would be 4.10. Since, however, it is a solution of salts, and especially of bicarbonates, the dissociation of the carbon dioxide would be depressed even below the above value. Moreover, Aberson has neglected to consider the influence of the salts present in the cell sap of the roots.

b. Hoagland's view.—The data of Hoagland *et al.* (**68, 70, 170**) would seem to afford evidence that CO_2 may not play an important rôle in *all* soils. These investigators found no measurable change in the reaction of soils of such markedly acid character of which the hydrogen-ion concentration is of the order of magnitude of the dissociation constant (3.0×10^{-7}) of carbonic acid (**200**), when such soils are partially saturated with carbon dioxide. In soils having a lower hydrogen-ion concentration (higher pH), however, the change in reaction due to partial saturation with CO_2 was quite significant. The decomposition of stall or green manures in *acid* soils of hydrogen-ion concentration of the above order of magnitude would, therefore, not be expected to increase the hydrogen-ion concentration of the soil to such an appreciable extent as to have any effect on the solution of "insoluble" materials. Inasmuch as there is an overwhelming amount of evidence from field and laboratory experiments that decomposing matter is a potent source of rendering the "insoluble" minerals of the soil available, the rate of decomposition varying with the age and nature of the material (**108**) and also nature of fertilization (**206**), we should—on the basis of Hoagland and Sharp's data—be forced to attribute the major favorable effect in the majority of soils not to carbonic acid but to the intermediate products of decomposition by the soil microorganisms, such as butyric, acetic and lactic acids—the dissociation constants of which are of the order 1.8×10^{-4} to 1.7×10^{-5}—and possibly also to replacement effects in the exchange complex.

Hoagland (**68**) sums up the situation as follows: "It appears to me that some soils are so acid that CO_2 is of slight effect, and that in other soils the nature of the soil minerals is such that CO_2 may have very little influence in promoting the solution of PO_4 or K; but, nevertheless, that CO_2 is of great general significance because of the existence of a great number of soils—not inconceivably the majority of soils—where CO_2 does appreciably alter the culture medium of the plant. In all this, we presumably must constantly keep in mind the difficulty of knowing just what the chemical system, and influence of CO_2, is at root-soil particle interphases." Yet it is evident from more recent field experiments (**166**) that there are exceptions even to this generalization.

The influence of nitrification processes must, however, not be underestimated (**187**). In soils rich in calcium, nitrification processes are generally accompanied by the destruction of bicarbonates, *i.e.*, with a decrease in the buffer value and increased utilization (p. 466). In practice, then, the aim should be made to increase the decomposition of organic matter without greatly increasing the calcium concentration of the soil solution. One way of doing this would be to apply lime in small amounts.

c. Experiments on the influence of artificial additions of carbon dioxide to the soil.—It might be expected that the results of experiments in which definite quantities of carbon dioxide are introduced into the soil in which plants are growing would give results from which definite conclusions could be drawn; but this is far from being the case. Lundegårdh (**103**) has shown the beneficial effects of increasing the carbon dioxide concentration of the atmosphere surrounding the external assimilatory organs, and Breazeale and Burgess (**11**) noted increased absorption of PO_4 by seedlings from "floats" when saturated with CO_2. The results obtained by Mitscherlich (**118**), Pfeiffer and Blanck (**145**), and also by Parker (**140**) are, however, decidedly negative with respect to its influence on the absorption of difficultly soluble materials; even distinct injury has resulted in some experiments (**118, 145**). These negative results, however, afford no proof that the carbon dioxide produced under field conditions does not play an important rôle in the assimilation of mineral nutrients. As Truog (**50**) suggests, the equilibrium conditions governing carbon dioxide evolution in the soil at the interface of the plasma membrane of the root hairs may not as yet have been duplicated in the laboratory.

It is unfortunate that all investigators have used either quartz sand or soils low in colloidal material in which the factor of ion replacement does not play the important rôle that it does in soils high in colloidal material. In view of the fact, moreover, that carbon dioxide is well known to have toxic effects when the CO_2/O_2 ratio is relatively high (**21, 24, 56, 118, 145, 167**), this possibility must also be considered in the experiments hitherto conducted on the effect of CO_2 additions. Pfeiffer and Blanck (**145**) observed distinct injuries, especially to oats, in their first series of wheat, lupine and oats cultures, when washed CO_2 gas was introduced at the bottom of the vessel for 10 minutes, 3 days a week at a pressure of 2.5 atmospheres. In their second series, when water saturated with CO_2 was used, no injury resulted; but even this procedure did not in Mitscherlich's experiments (**118**) prevent injury to the plants.

In none of the experiments on the effect of the artificial addition of CO_2 is the total concentration of carbon dioxide in the soil at any definite interval of time known. The possibility of toxic doses, therefore, is not eliminated. Thus, in Parker's experiments (**140**) the concentration of carbon dioxide was sufficient to increase markedly the absorption of silica. Evidence exists that different plants differ in sensitivity to CO_2 (**45, 179**), although in the culture solution experiments of Hall (**56**) wilting occurred when CO_2 was aspirated through all culture solutions. Stoklasa (**182**) and also Lundegårdh (**102, 103**) found that a concentration as low as 1 per cent. of CO_2 in the soil atmosphere 15 cm. below the soil surface retarded growth, and was even toxic to some plants.

It is of interest to note here that Pfeiffer and Blanck (**145**) argue from the negative results obtained by CO_2 additions in their "oder-sand" cultures in the presence of rock phosphate that the data afford proof that other acids are exuded by roots, because, although many of the plants with which they experimented were able to utilize rock phosphate quite well, the artificial addition of CO_2 gave in all cases decreased yields.

3. Root excretions other than CO_2

a. The divergent views.—The tendency of the European workers (**1, 28, 55, 120, 183**) and also of some of the American investigators (**52, 67, 68**) is to emphasize the importance of carbon dioxide exudation, and to regard the rôle of other acid exudations, relatively, of little significance, and, indeed, in some cases (**52, 55, 183**) to question their existence. Others (**28, 120**) have claimed to have found substances other than acids in the root exudations.

b. Distinction between excretions from living and dead cells.—Although the fact that acids may be excreted from dead cells was early recognized by Czapek (**28**), it may be questioned if, in planning some experiments (**12**), sufficient attention has been paid to the necessity for making conditions such that the excretions of living and dead cells could be differentiated. That this point is of importance is shown by the results of Johnson (**75**), who found marked differences in the quantities of Ca^+ and Cl^- absorbed from a $CaCl_2$ solution, in the case of live and dead beet cells. The dead beet cells removed only 26.2 per cent. Cl from the roots whereas living beet cells removed 43.7 per cent.; but the proportion of Ca absorbed was nearly the same in both cases.

Osterhout (**133**) and also Rudolfs (**160, 161**) have also called attention to the effect of hydrolytic dissociation of neutral salts and to the effect of the differential rate of ionic absorption. These secondary effects, due to differences in the permeability of living and dead cells, will be discussed later in this paper.

c. Root sap acidity as a basis for chemical methods of determining nutrient requirements.—(1) *The direct method of approach.*—The assumption that acids were exuded from roots served as a basis for the attempts of Dyer (**38**) and his school to ascertain the fertilizer requirements of soils. Dyer argued that if acids are exuded, the total acidity of the sap should be an approximate measure of its solvent action. The sap acidity was obtained by averaging the results of determinations for total acidity of the water extracts of the roots from over a hundred different species of plants. This was found to be equal approximately to a 1 per cent. solution of citric acid. Lemmermann (**96**) also adopted a similar criterion and, as we shall see later, the greater assimilatory ability of the Leguminosae compared with the Gramineae was attributed by him to the greater total acidity of the root sap of the former.

Information on the nature of the root exudations was also sought by Czapek (**28**) and Kunze (**89**) by bringing intact seedling roots in contact with litmus paper and noting the change in color. It has been suggested by Haas (**54**) and others, however, that it is not possible to reach definite

conclusions from this type of experiment because different roots may differ in their capacity to absorb the blue dye of litmus. Differential absorption cannot account for all the facts, for Kunze (**89**) showed by titration in parallel experiments that the acidity exhibited was far greater than that indicated by the turning point of litmus. And there is no evidence in Kunze's experiments that the acids arise from injured or dead cells. It is clear, too, that Kunze recognized the limitations of litmus paper as an indicator, for he states that in some plants the amount of acid lies below the sensitivity of litmus.

Czapek (**28**) attributed the red color obtained by pressing seedlings against blue litmus paper to the exudation of KH_2PO_4, and emphasized the fact that the exudation of this salt could not be explained by any injury to the cells of the root hairs because the same result was obtained in culture experiments where there could be no question of injury; but it is to be noted that Czapek did not find the drops of liquid—always to be observed on the roots of seedlings developed in a humid atmosphere—to be acid. Prianishnikov (**153**) also found traces of PO_4 in the root secretions of *seedlings, but not from full grown normal plants.*

The whole subject of the secretion of acids other than CO_2 was subjected to a critical investigation by Stoklasa and Ernest (**183**) with *Hordeum vulgare* and *Zea mays*, using a different technique. The seedlings were allowed to develop for 15 days in distilled water and then transferred to experimental cylinders, through which 20 liters of sterilized air free from CO_2 were passed. In one series of experiments a mixture of O_2 and N_2 gas was used. Under these conditions CO_2 and a trace of H_2 was found, but no PO_4 nor SO_4. In view of the fact that a large number of investigators (**8, 13, 79, 94, 101**) had reported the presence of lactic, acetic and formic acids in the root exudations of plants, Stoklasa and Ernest carried out a similar series of experiments from which they conclude that these acids were only exuded when the root system suffers from lack of oxygen and is the result of partial intramolecular respiration. When the supply of oxygen was sufficient only carbon dioxide was found. These experiments were carried out at a uniform temperature (20–22° C.) and observations were made for 10–20 days. Air containing α-radium emanations increased respiration considerably.

Stoklasa and also Hall (**55**) have argued, from an ecological standpoint, that it is improbable that such important nutrients as K and P_2O_5 would be exuded from the root system; but this position is untenable, for many experiments (**15, 207**) show very clearly that plants may return to the soil at some stage of their growth, usually at maturity, some of these important nutrient elements.

(2) *The indirect method of approach.*—Some investigators (**117, 145, 181, 192**), on the other hand, have sought a solution of the problem of acid exudation of roots by determining the assimilatory power of different plants for difficultly soluble materials, like rock phosphate, and Hall (**55**) has sought a solution by taking out a balance sheet between the acid and bases contained within the completely developed plant.

(a) *Mitscherlich's view.*—The conclusions from the indirect methods devised by Mitscherlich (**119**) and that adopted by Pfeiffer and Blanck (**145**) are contradictory. Mitscherlich (**119**) maintains that his "Wirkungsfaktor" values are a constant characteristic for each manure (fertilizer) on all crops and all soils. This "law" of Mitscherlich is expressed by the equation

$$\frac{dx}{dy} = k(A - y) \tag{7}$$

$$\text{or } \log_e (A - y) = \log_e A - c(x + b) \tag{8}$$

where: c = "Wirkungsfaktor" or action produced by the nutrient element applied,
A = maximum yield when x is not limiting,
x = amount of nutrient element added,
b = amount of available nutrient already present in the soil,
y = yield obtained by application of nutrient element x.

He argues, therefore, from the constancy of c that all plants must have at their disposal the same agent for the decomposition of difficultly soluble material and that this is CO_2 exclusively. In recent years he appears to have changed his views to some extent as a result of the following experiment (**120**), which is easily carried out. When a very dilute solution of HCl (0.0001 N) is added to root hairs observed under a microscope, at first local swelling up of the protoplasm can be observed, followed by a rupture and expulsion of the protoplasm into the surrounding medium. After some time *new root hairs are formed*, showing that the injurious effects of too high hydrogen-ion concentration have been overcome. These phenomena may, in part, be explained by the tendency of plants to change the reaction of the medium in the direction of neutrality as observed by Pantanelli (**138**), Hoagland (**64, 65, 67**) and Theron (**185**). The medium might be neutralized by the absorption of hydrogen-ions and the excretion of OH ions simultaneously, or, as Mitscherlich believes, the injurious effect of the acid may be counter-balanced by the liberation of substances of the nature of organic buffers. These ideas would coincide with Mazé's (**109**) and also Comber's view (**26**) that substances other than those of acid nature take part in the solubilization of mineral nutrients. That considerable organic matter is liberated from the root sap and root hairs sloughed off by plant roots is shown by the observations of Lyon and Wilson (**105**), but these findings are not applicable as evidence to the exudations of intact root hairs. Minima's investigations (**116**) afford some evidence, although indirect, that organic acids are excreted by roots, on the basis that the period of maximum excretion of non-volatile organic acid buffers is coincident with that of maximum acidification of the soil.

Mitscherlich, in his earlier papers (**118**), held the view (**146, 147**) that the amount of P_2O_5 absorbed from rock phosphate by oats was identical with the amount dissolved by a saturated solution of carbon dioxide, and concluded (**117**) that the different "Aufschliessungs-vermögen" of different plants could be explained by differences exhibited in their ability to develop root systems, which, in turn, is proportional to the amount of carbon dioxide respired by them. More recently he has modified these views and has discarded chemical methods as being inadequate (**119**). Indeed, it has been found by numerous workers, especially by König (**82**) and Schloesing (**168**), that oats and other plants may absorb far more P_2O_5 from rock phosphate and other soil phosphates than can be dissolved by a saturated solution of CO_2. Frear and Erb (**43**) also drew similar conclusions with respect to the soil K_2O of the fertilizer plots of this Experiment Station.

In evaluating these opposing claims, it should be remembered that Stoklasa (**180**) and Stoklasa and Ernest (**183**) found that lactic, acetic, formic and oxalic acids were excreted only when the root system suffers from lack of oxygen; but as yet there are no critical experiments indicating whether or not under average field conditions, the system in immediate contact with the roots may not be relatively deficient in oxygen. The accumulation of these organic acids due to incomplete oxidation of the products of respiration has been found by Stoklasa to be highly toxic to plants. This view is further supported by Mazé's experiments (**109**) with peas in sterile cultures. That the problem of air renewal is a serious problem, especially in some of the finer textured soils, is apparent from technical remedies such as drainage and crop residues devised to meet it.

(b) *Hall and Miller's experiments.*—One of the arguments used by Hall (**55**) upon which to substantiate his conclusions that only CO_2 is excreted by the plant is based on the findings of Hall and Miller (**57**) that the net action of the plant upon the soil is to leave an excess of base in the soil. This result was arrived at by taking a balance sheet between the acid and bases contained within the completely developed plants (wheat, barley, sweeds and hay) of the Rothamsted rotation experiments on the Agdell field, and also of wheat and cabbage plants grown in culture solutions. These experiments, however, do not take into consideration the effect of hydrogen-ion concentration. As Theron (**185**) and others have emphasized, greater equivalent proportions of anions than of cations are absorbed on the acid side and on the alkaline side the reverse holds true. If Hall's statements had been made later, they undoubtedly would have been modified as a result of the recent progress in physical chemistry. Furthermore, the validity of the assumption made by Hall that all the nitrogen in the plant should be calculated as an acid on the basis that all the nitrogen enters the plant as nitrate may be questioned.

(c) *Kappen's experiments on expressed root saps.*—The procedure of Kappen (**77**) of determining the total acidity and hydrogen-ion concentration of the expressed sap of the roots of mature plants grown in field experimental plats is more pertinent; but even in these experiments values cannot be regarded as absolute, because—as we shall see later—of the existence of a hydrogen-ion concentration gradient (**53, 195**). Moreover, Kappen's experiments are open to the criticism that they do not distinguish between diffusion products and exudations of the roots. Considered, however, from the standpoint of the relative comparisons between total acidity and hydrogen-ion concentration of the respective root saps, Kappen's data indicating low hydrogen-ion concentration values but high titration numbers are instructive. Only 0.1 per cent. of the hydrogen-ions were found to be present in the ionic condition, the remainder being in the undissociated form. The inevitable conclusion is that the root saps must consist of a mixture of free organic acids and their alkali or alkali-earth salts. This point is of interest because such an arrangement would ensure the organism against too violent fluctuations in the degree of acidity of its sap, and is analagous to the conditions that exist with other physiological fluids, blood, urine, etc. Both Groh (**49**) and Pavlinova (**143**) come to the same conclusion as Kappen, but the deductions of the latter, unfortunately, are of limited application because only seedlings were used. They are of interest, however, in that they show that the older technique adopted by Czapek (**28**) is useless to determine the reaction of the water exuded by guttation.

(d) *Haas's experiments.*—Haas (**52**) sought a solution of the problem of root exudation by determining simultaneously the hydrogen-ion concentration of solutions in which seedling plants were growing and also in controls without plants. After the CO_2 had been expelled by a current of pure H_2, the hydrogen-ion concentration of both series of cultures was identical. Although this method eliminates the possibility of confusing the exudation of living cells from those of dead cells, it is doubtful if the conclusion drawn by Haas from these experiments, *viz.*, that plants do not exude other acids than CO_2, can be extended to *mature plants*. The mineral nutrient requirements of the 10 day seedlings with which Haas worked are, of course, much less than in more mature plants.

C. The influence of the degree of the acidity of the cell sap

In view of the emphasis placed by many investigators upon the influence of the degree of the sap acidity of cells as a determining factor in the feeding of plants, it is pertinent for us to inquire more closely into this phenomenon.

1. Does a hydrogen-ion concentration gradient exist

Although there is sufficient evidence to indicate that the hydrogen-ion concentration of the substrate has an indirect influence on the growth of plants, it is not definitely known what, if any, relation exists between the hydrogen-ion concentration of the medium and that of the metabolically active tissues. Jacobs (**73, 74**), in the case of isolated cells, found none. Hoagland's results (**66**) on barley grown in water, in sand, and also in soil cultures indicate that the pH values of the sap of the tops were almost identical, even though the pH of the nutrient media varied widely, *viz.*, from 4.94 to 6.76 in water cultures and from 7.03 to 7.34 in soils. Reed and Haas (**157, 158**) report similar results from water culture experiments. The latter (**157**) and also Theron (**185**) have obtained comparable results in the case of the sap of tops, but in the roots Theron (**185**) noted that the pH increased with increase in pH of the medium (substrate). The existence of a definite hydrogen-ion concentration gradient in the case of algae has been shown by Child (**23**) and in some of the higher plants, grown in soils, by Haas (**53**) and in animal tissues by Gustafson (**51**). The regulatory influence of buffer systems, therefore, must vary considerably in different parts of the plant. The data of Haas (**53**) are illuminating. In *Melilotus alba* (second year growth), the following pH values were obtained: Soil extract, 7.68; root (6″ portion below upper 2″), 5.82; root (2″ of upper part), 6.46; leaves and petioles, 7.04; stems, 6.68. The pH of the mixed upper 3″ of the tops, stems, leaves and buds was 8.0. As Reed and Haas (**158**) later pointed out, any hypothesis based upon differences of acidity of the plant sap and the external medium must account not only for the accumulation of inorganic elements within the roots but also for their movements to other portions of the plant. Investigators in this field must, moreover, take cognizance of the fact that marked change in the hydrogen-ion concentration usually occurs within an hour after expression of the plant sap (**25, 50, 61, 195**).

From the foregoing, it is apparent that we are not yet in possession of sufficient evidence to determine whether or not the cell sap of tissues possessing similar functions have hydrogen-ion concentration values that may be characteristic of the species. The assumption that root sap acidity values are a characteristic of the species formed the basis of much of the experimental work by the German investigators, (**1, 89, 96**) in their search for a solution of the causative factors determining the "Aufschliessungsvermögen" of different species. Thus, the Gramineae and the Leguminosae were supposed to be sharply differentiated (**96**) on the basis of the acidity of their root sap, until Kappen (**77**) and later Stoklasa (**181**) showed very definitely that the distinction held neither for the titration nor hydrogen-ion concentration values. Stoklasa's experiments (**181**), if confirmed, would lead to the conclusion that the acidity of the sap of the roots of all cultivated plants is near neutrality, except when insufficient oxygen is present, under which latter condition the acidity increases. He emphasizes the significance of this fact for the development of the bacteria of the rhizosphere.

As we shall presently see, working on this assumption, Truog (**194**) considers that the feeding power of different plant species is a function of the different intensities of the acidity of the respective saps. Thus, buckwheat and clover are cited as types of plants with high and low acidities, respectively. The table below, however, taken from the data of Truog and Meacham (**195**), Kappen (**77**), Haas (**53**), Stoklasa (**181**), and Aberson (**1**), will show how impossible is such a classification, at least with the methods hitherto adopted.

TABLE III

pH OF BUCKWHEAT AND RED CLOVER ROOTS REPORTED BY SEVERAL INVESTIGATORS

Investigator	Buckwheat	Red clover
Truog and Meacham	4.0 – 4.03	5.6 – 5.9
Kappen	4.9 – 5.3	6.2
Aberson	5.7 – 5.9	7.9 – 8.4
Haas	4.8 (mature plant) 5.4 – 5.9 (seedlings)	5.8 – 6.1
Stoklasa	6.2	6.6

Further development of Němec's work (**125**) on the hydrogen-ion concentration of the sap of seeds of a large number of different species of plants promises—from the practical standpoint—to clarify the whole situation. Seeds are more nearly constant in composition than other parts of plants, and Němec found that the hydrogen-ion concentrations of the seeds of plants adapted to acid soils are relatively much higher than those adapted to alkaline soils. It is of interest to note that the saps from the seeds of barley, corn and peas were found to have the lowest hydrogen-ion concentration, pH 6.45 – 6.30.

It has been a matter of long observation that such plants as sweet clover, alfalfa and lettuce are adapted to grow on soils at or near the neutral point. When soils to which these plants are adapted are allowed to go unlimed, they are replaced by others that are less sensitive to acid conditions. Camargo *et al.* (**19**) have recently shown in controlled experiments that the optimum growth of roots of the coffee plant is at pH 4.2, which affords an explanation of their adaptability to acid soils. The coffee plant is greatly injured by even moderate liming.

D. Theories proposed to explain differences in the feeding power of plants

1. The application of the law of mass action

Tschirikow (**196, 197**) stressed the importance of the CaO/P_2O_5 ratio in the plant as an important factor in the ability of different plants to supply themselves with enough P_2O_5 from difficultly soluble phosphates. Plants having a high energy absorption for Ca would, according to him, depress the Ca concentration of the soil solution, resulting in an increased solubility of the phosphate. The smaller the amount of P_2O_5 necessary for the formation of one unit of organic substance of the plant (*i.e.*, the smaller the percentage of P_2O_5 in the plant), the greater obviously would be the effect of P_2O_5. From this viewpoint the capacity of the plant to absorb difficultly soluble phosphates is directly parallel to the quantity of Ca absorbed and inversely parallel to the P_2O_5 requirement of the plant.

Truog (**192, 194**) has extended Tschirikow's views further and has invoked the concept of equilibrium between calcium phosphate and carbonic acid, on the one hand, and the products of their interaction, on the other, as expressed by the equation:

$$Ca_3(PO_4)_2 + 2H_2CO_3 \rightleftarrows Ca_2H_2(PO_4)_2 + CaH_2(CO_3)_2 \qquad (9)$$

He contends that plants such as buckwheat, which the majority of culture experiments show is capable of utilizing the phosphorus of rock phosphate in large measure, are supposed to be able to absorb both of the products to the right of the equation. It is to be inferred, moreover, as already stated (p. 462), from Truog's later expansion of this view that the rate of absorption is determined by the degree of acidity of the saps of these plants. Thus, buckwheat is characterized as a plant with "high internal acidity." In the case of plants having a low degree of sap acidity, one of the products to the right of the equation (calcium bicarbonate) is assumed to be incapable of absorption by the plant at a rate comparable with that of the other product (di-calcium phosphate); consequently, the operation of the reverse reaction will prevent the further solution of the rock phosphate. Corn and oats are placed in this class by Truog.

This view has been supported (**6, 7, 53**) and as vigorously disputed (**30**). In order to clarify our ideas, it should at the outset be remembered that, although the possibility (p. 456) of absorption of salts in the undissociated form (**133, 135, 137**) must be considered possible under some conditions, *e.g.*, in the case of detached cells, the weight of evidence (**67**) is that absorption in normal intact plants takes place in the ionic condition. Now there is ample evidence, as we shall see later, to show that not only do root hairs of different species differ in their degree of permeability to the same ion, but that the different relative velocities of the anion and cation of a salt may also be a factor in determining the equilibrium. We must then be prepared to consider ionic equilibrium, although the existence of such equilibria between the ions in the cells and the ions in solution has not as yet been established definitely.

2. Evidence for and against Truog's views

a. Bauer's sand culture experiments.—The results of Bauer (**6**) have been cited in support of Truog's views. In these experiments corn was grown in sand cultures treated with rock phosphate (or acid phosphate) as the source of P, and $NaNO_3$ or $(NH_4)_2NO_3$ as the source of N; but the analytical data are insufficient to show that the improvement in growth in the leached pots was due to removal of calcium as the bicarbonate. Removal of toxic substances by leaching must also be considered a factor (**119**). Further critical experiments on this point should include pots without plants as controls.

b. The views of the California group.—Davis, Hoagland, and Lipman (**30**) cite Newton's (**126**) results with peas and barley in water culture solutions, which grew normally without bicarbonates, and, moreover, in solutions at hydrogen-ion concentration at which bicarbonate ions could not exist to any extent. But this argument may be weakened by the fact that Hoagland (**67**) and others have later deduced evidence to show that rapid replacement of $-HCO_3$ ions by other ions may occur.

Reed and Haas (**157**) have also argued against Truog's view on the basis of their experimental work with walnut and orange trees. In experiments with the former the effect of a solution of $Ca(OH)_2$ of pH 9.0 was determined. Although the percentages of Ca in the ash of the tops and roots were 14.60 and 9.80, respectively, the pH of their sap (5.26–5.48) showed no significant changes from values obtained when such plants were grown in complete culture solutions. This same argument, however, used by Reed and Haas to refute the existence of a relation (**193**) between the absorption of calcium and the degree of acidity of the sap, breaks down completely when certain experimental data (**78**) may be interpreted, by the same method of reasoning, to prove that calcium may be necessary for precipitating organic acids formed as a by-product, on the basis that mottled leaves of citrus trees starved of calcium are considerably more acid and contain distinctly less calcium than normal leaves.

Again, Reed and Haas (**158**) are inclined to the belief that there is no evidence in support of the assumption by Truog (**194**) that absorbed materials are converted into insoluble compounds as rapidly as they accumulate in the plant, or that absorption *necessarily depends* on the precipitation of ions within the plant. The italics are the writer's. But this conclusion is not justified from the experimental data supplied. Thus, Reed and Haas (**158**) find that in sand cultures the concentration of water-soluble K increases in the ash of leaves, trunk, shoots and stems, and that the concentration of soluble Ca increases in the leaves *but decreases* in the shoots, trunk and roots as the concentration of the nutrient solution increases. In soil cultures 45.82 per cent. of the Ca was soluble in the leaves, 15.03 per cent. in the shoots, and only 7–9.5 per cent. in the trunk, roots and rootlets. In buckwheat nearly all the calcium is insoluble in water (**67**). The fact that *Nitella* (**69**) contains large quantities of soluble potassium and calcium salts has also been used as an argument (**30**) against Truog's views. But to what extent the extension of data obtained from such abnormal structures as *Nitella* to the higher cultivated plants is justifiable remains to be determined.

The work of Kostychev and Berg (**88**) has an important bearing in this connection. They have determined the forms in which Ca is present in the tissues of a number of plants. The following interesting data, table IV, are taken from their paper:—

TABLE IV

Forms of calcium in plants

Plant	Percentage of the total Ca		
	Soluble in water	Combined with PO_4 and HCO_3	Ca oxalate
Trifolium repens (leaves)	42.4	38.6	19.0
Alchemilla sp. (leaves)	35.6	40.3	24.1
Caragana arborescens (leaves)	21.0	23.1	55.9
Solanum tuberosum (leaves)	16.5	34.5	49.0
Lemna minor	11.6	45.7	42.6

In apple tree experiments (**190**) the writer has found in mature leaves relatively low percentages of the total Ca soluble in water, *viz.*, 17.2 per cent., and relatively high percentages as the oxalate, *viz.*, 54.3 per cent. The above data might, therefore, be just as readily used to support Truog's views as to refute them.

Chibnall (**22**) has recently shown that one-tenth of the water-insoluble calcium of cabbage leaves consists of calcium phosphatide.

c. Some weak points in Truog's theory.—

(1) *The experiments of Parker—Bauer—Bartholomew.*—According to Truog (**194**) plants with a slightly acid or neutral sap, like sweet clover and alfalfa, are more able to utilize the potassium advantageously from lower concentrations of the element than plants, like buckwheat, having a more acid sap; but Parker (**139**) found that buckwheat absorbed relatively large quantities of soil potassium. Moreover, Bauer (**6**) found that both buckwheat and sweet clover readily absorbed calcium from rock phosphate. Bartholomew (**4**) also failed to find any relation between the amount (percentage) of calcium absorbed and the ability of these plants to feed on rock phosphate, although the substrate and nutrient medium were the same as that used by Truog (quartz sand).

(2) *The effect of the destruction of the buffer systems.*—No account has been taken of the buffer systems present, the destruction of which must be of greater influence than the factors already noted. The buffer action of calcium bicarbonate, for example, is a function of the hydrogen-ion concentration of the medium, and is readily destroyed if the hydrogen-ion concentration varies from pH 7.5.

$$Ca(HCO_3)_2 + NaOH = CaCO_3 + NaHCO_3 + H_2O \qquad (10)$$

$$Ca(HCO_3)_2 + H_2SO_4 = CaSO_4 + 2H_2O \quad + 2CO_2 \qquad (11)$$

The destruction of $Ca(HCO_3)_2$ results, as shown by Butkewitsch (**16**), in increased yields—in some cases as much as three times—and the amount of calcium in the medium decreases. The stronger the buffer action the greater the CaO/P_2O_5 ratio. From this standpoint, the inhibitory action of $Ca(HCO_3)_2$ on phosphate utilization may be regarded as that of paralyzing the root activity of the plant. *In this sense and in this sense only may* Truog's *view hold.*

(3) *Do the terms "high" and "low" acidity have any meaning?*—The validity of Truog's conception must necessarily rest also upon the accuracy of his classification of the relative acidities of the saps of plants into high, medium and low; but, unfortunately, we have no definite knowledge of the relative acidities of plant saps. As we have seen, the variation in different parts of the same plant is so great that it is not, as yet, justifiable to use the terms "high" and "low" in classifying plant saps. The hydrogen-ion concentration of the sap of seeds is much more constant; therefore, in any attempt to correlate hydrogen-ion concentration of plant tissues with nutrient supply, the examination of the sap of seeds would be the most profitable line of attack.

d. Lack of harmony of observation with theory.—Nevertheless, it is highly improbable that the results upon which Parker and Truog (**142**) based their classification of plants into (1) high Ca and high N, and (2) low Ca and low N are accidental, as has been contended (**129**). On the contrary, the preliminary studies of Storck and Rippel (**134**) support the existence of such a relationship between Ca and N. True, the analytical data presented by Parker and Truog represent the composition of plants grown under very varied and different conditions. But an examination of the paper (**142**) will show at once that the data have been collected with great care and, moreover, Truog has himself conducted carefully controlled experiments. He recognizes (**193**) that isolated analyses may be found that are relatively quite different from those used—especially of plants that have reached maturity or were cut as hay—due to losses of Ca, in part possibly by leaching (**95**).

If we accept this relationship between calcium and nitrogen as an actual fact, to what may it be attributed? Surely not to acids arising from the decomposition of proteins, as Truog assumes. The older physiologists (**33**) considered that "living" protein substance of the protoplasm functions as the immediate material for respiration, resulting in the production of the acids found in plants. But, as pointed out by Kostychev (**87**), this assumption contradicts an abundance of facts that have been established recently without question—that sugars are much more easily oxidized than proteins. Most of the plant acids arise from the carbohydrates and fats that serve as the fundamental sources of energy of respiration (**156**). As a matter of fact the amount of energy obtained by the decomposition of proteins is very small, and, moreover, the amino acids formed are readily converted by the plant again into proteins (**176**). Only after the complete consumption of sugars do plants begin to burn the protein material of their protoplasmic framework, and then the CO_2/O_2 ratio falls to 0.70–0.80.

The explanation of the Ca/N relations observed by Parker and Truog (**142**) is more probably to be explained by the antagonistic action of Ca and K resulting in an increased absorption of N and P_2O_5 (**188**).

e. Extent to which bases function as neutralizers of plant acids.—Truog and Meacham (**195**) and also Haas (**53**) have examined the hydrogen-ion concentrations of the tops of a large number of plants grown both in limed and unlimed plots, and, without considering the possibility that the results are within the limits of experimental error (**185**), it was found that with few exceptions the pH of the tops of the *limed* plants was higher than that of the *unlimed*. It might be argued from these facts (**53**) that one function of calcium is to adjust the pH of the soil solution so as to enable the plant to secure a sufficiently rapid supply of bases to neutralize the injurious effect of too high acidity; but, apart from the fact that neither CLEVENGER (**25**) nor DUSTMAN (**37**) have been able to corroborate such a conclusion, there is absolutely no justification to assume that this function is more peculiar to calcium than the other bases, *e.g.*, magnesium and potassium (**90, 91**). Moreover, it is not necessary to assume that the disappearance of acids is accomplished solely by the mechanism of neutralization, since ASTRUC (**2**) has shown conclusively that the disappearance of acids—which are always higher in the young plants—is due not so much to their "neutralization" as to the process of respiration and esterification.

f. CALCIUM REQUIREMENTS OF PLANTS ARE VERY DIFFERENT.—It is certain that plants cannot continue growth without calcium and, although different species respond differently to its presence, injury does not result if sufficient quantity of soluble salts are present in the media (**157**). The observation of LOEW (**114**), therefore, of the death of cells on treatment with salts that precipitate Ca must be interpreted accordingly.

The response of species to varying quantities of calcium appears to be an important ecological factor (**201**). Thus, SKEEN (**175**) has shown that *Phaseolus vulgaris nanus* must have large amounts of calcium and that *Lupinus albus* obtains its maximum growth with relative traces.

From the foregoing, we are forced to conclude that, although the suggested explanation (p. 467) offered to account for this Ca/N relationship may not explain all the facts, there is no sound reason for denying the existence of this relationship. It is unfortunate, from this standpoint, that the nitrogen content of the oat plants grown by SHEDD (**171**) on different soils and which showed such extreme ranges in calcium content are not known. This would have given us some valuable information.

3. CONCLUSIONS FROM RECENT MORE DIRECT EXPERIMENTS

RUSSELL (**162**) considers that the critical problem is to know whether the concentration of the substrate (nutrient solution) with respect to calcium has decreased to a greater extent in the "high feeding" plants than in the "low feeders." Such experiments are at present being carried out by DOMONTOVITSCH and SCHESTAKOW (**34**), of which only the preliminary results have been published to date. In sand culture experiments with lupines, buckwheat, mustard, millet and oats—using rock phosphate (size of particles 0.1 mm.) as the source of P—the growth of the plants and concentration with respect to P_2O_5 and Ca were compared between pots containing mixed plants, *viz.*, (1) buckwheat and oats, (2) lupines and oats, (3) lupines and millet, (4) mustard and oats, and pots containing an equal number of plants grown alone. The following table V gives the results of the preliminary work:—

TABLE V

DATA FROM DOMONTOVITSCH AND SCHESTAKOW

NR. DER GEFÄSSE	P_2O_5—QUELLE UND PFLANZENART		pH	P_2O_5 IN 100 CC. DER LÖSUNG MG.	CA IN 100 CC. DER LÖSUNG MG.
4	Rohphosphat	Hafer	7.43	0.020	95.0
16	"	Hirse	7.14	0.018	111.93
6	"	Buchweizen mit Hafer	5.43	0.041	....
5	"	Buchweizen	6.25	0.036	....
11	"	Lupine	5.34	0.767	30.85
12	"	Lupine mit Hafer	5.17	0.679	75.89
17	"	Lupine mit Hafer	4.80	1.350	48.59
26	"	Hafer	7.21	Spuren	....
36	"	Senf mit Hafer	6.35	grössere Spuren	....

The growth of oats and millet was scanty and not much greater than those plants without any phosphorus addition. In the mixed cultures with lupines the yield of oats and millet was increased 545 and 846 per cent. respectively, over the yields of these plants grown alone. Mustard and buckwheat also influenced the growth of these Gramineae to a remarkable extent. Unfortunately, calcium determinations are not reported in all cases; but it is evident that the mixed cultures with lupines have reduced the concentration of the calcium under that in the pots in which the Gramineae grew alone. The hydrogen-ion concentration of the medium in the mixed cultures is also much lower than that in the cultures of plants grown alone. These results indicate that the differences in the utilization of rock phosphate in these experiments are to be correlated with their effect on the hydrogen-ion concentration of the root medium and, perhaps, also with the unequal rates of absorption of calcium. The data for concentration of P_2O_5 show no relation between the rates of absorption of phosphate ion by the different plants.

HOAGLAND has recently reported that with the same source of rock phosphate, buckwheat was able to make normal growth, whereas tomato plants were almost entirely unable to obtain phosphate from this source—yet the tomato plants had a much higher percentage of calcium than the buckwheat plants (**68**). The conclusion is drawn that the solid phase of the soil and its ability to keep the soil solution supplied with calcium is an important factor. Results obtained by MAZÉ (**109**) may be similarly interpreted. It would be of interest for investigators in this country to conduct experiments similar to those of DOMONTOVITSCH and SCHESTAKOW's work and, in addition, to determine the content of the plants in Ca and PO_4. It is well to bear in mind, however, the possibility that the carbon dioxide exuded by roots may, under the artificial conditions of many of the experiments reported, be of far greater importance than under normal conditions and for this reason it is undoubtedly more advantageous to use soils as the substrate.

E. The influence of the extent of the root system

1. Is there any relationship between feeding power and extent of roots

That great differences exist with respect to the extent of the root system among different types of plants is a common observation. A review of the literature has been given by Miller (**115**). This difference in the growth of plant roots outwards, together with the differences in their respiration energy, is regarded by some authorities (**30**) as an important factor in the mechanism of absorption of ions by plants.

Truog has also clearly recognized that differences in the extent of root system may be a factor in the absorbing power of plants. This is quite evident from a statement in one of his earlier papers (**193**) to the effect that "the feeding power of a plant for lime depends largely on the extent and character of the root system." From the results of experiments carried out later, Truog modified this view and, as we have seen, stressed the internal acidity of the sap as a more important factor in plants like buckwheat, which have a very restricted root system.

But there are several experiments (**84, 152, 154, 155, 192**) that indicate no evidence of the existence of any relationship between feeding power and the extent of the root system. In quartz sand cultures with rock phosphate as the source of phosphorus (**84, 152, 154, 155, 192**) much larger yields and a much higher percentage of P_2O_5 were obtained in the case of the legumes (peas and lupines) and also of buckwheat than of the cereals. In other words, in these experiments buckwheat—with a small root system—(**145, 146**) utilizes rock phosphate better than the cereals which have, relatively, a very extensive root system. Parker's data (**139**) are of interest in this connection. Comparing the absorption of sorghum and cotton under similar conditions, it was found that the latter absorbed more than double the amount of ash constituents than the former. If, as the majority of field observations tend to indicate, sorghum has a more extensive root system than the tap-rooted cotton plants, differences in absorption of mineral constituents cannot, in Parker's experiments, be attributed to differences in extent of root systems. On the other hand, Davis *et al.* (**30**) cite Newton's experiments (**126**) to show that the extent of root system is an important factor in the absorption of ions from soil. This conclusion is based on Newton's experiments in which peas and barley were grown together. Under these conditions the peas absorbed more calcium than barley from *soil cultures*; but there was no similar difference in *solution cultures*. It is argued, therefore, that if barley and peas differed considerably in extent of root system such results would be expected to follow. Data on the growth of the respective root systems in these experiments are not given. In a later experiment (**128**) on the evolution of CO_2 from barley and peas, the dry weights of the roots of peas were found to be less than those of barley. Newton himself (**128**), however, is inclined to attribute these differences to the much larger respiration energy of peas over barley than to differences in the extent of root systems. But Hoagland (**67**) later pointed out that comparisons of extent of root systems have only a very limited value, because the general appearance and size of a root system is not necessarily an accurate index of the total active absorbing area. In this connection it is of interest to note that Russell (**162**) is of the opinion that the whole question of the supply of nutrients involves both root spread and replacement of ions, although no experimental evidence is cited.

Consequently, views on the relation of extent of root system to feeding power are necessarily speculative until more critical data are forthcoming. The method of determining the actual absorbing surface such as that employed by Dustman (**37**) and others is unquestionably the most accurate. A critical discussion of the relative merits of the various procedures has been given recently by Weaver and Himmel (**202**).

F. The influence of other factors

1. Permeability

In an earlier paper (**187**) the writer has discussed some of the factors involved in "membrane" permeability. Until sufficient quantitative and qualitative data, obtained under rigid control of all the variables are available it is improbable that views on the subject can be anything but speculative. Possibly, a satisfactory working hypothesis may be formulated only when data, based upon cataphoresis experiments, have accumulated from a wide variety of materials carrying no charge (**99**). The importance of securing more qualitative information is indicated by the interesting properties of the phosphatide ($RO_2 \cdot C \cdot CH_2OH(C \cdot R'O_2)CH_2O \cdot PO_3H_2$) isolated by Channon and Chibnall (**22**). It forms a fat-soluble calcium salt but a water-soluble sodium salt; and, accordingly, cell permeability could be conceived to be altered by a change in the proportion of calcium to sodium salts.

From thermodynamic considerations and also from the kinetic theory it can be deduced that ionic pairs having the higher mobility will pass through a membrane in proportionally the greater quantity, and also that the permeability of an ion is increased by the introduction of another electrolyte possessing a common ion. Thus, many investigators (**100**) have found that the absorption of K from KCl is increased by the addition of NaCl; and the effect of the NaCl has been shown to be dependent on the hydrogen-ion concentration of the medium. Since hydrogen and hydroxyl ions have the greatest velocity, the addition of acids (decrease of pH) will increase the diffusion of anions; the addition of alkalies (increase of pH) that of cations (**46, 47, 48**).

The influence of the hydrogen-ion concentration of the medium on the absorption of *weak acids and bases and their salts* by living cells has been investigated by Jacobs (**73, 74**). The pH of the medium to which a cell is exposed was found to bear no necessary relation to the pH produced by that medium within the cell itself. The higher permeability of living membranes to undissociated acids and bases results in a higher physiological activity of weak acids and bases compared with stronger ones. Conditions favoring the formation of undissociated molecules, therefore, will promote their penetration into membranes to which they are permeable. The result of these influences may, as Loeb (**100**) has shown, be to modify the properties of the cell wall in such a way as to accelerate the rate of diffusion of certain ions and retard the rate of diffusion of other ions. $CaCl_2$ and NaCl are important factors in causing such selective diffusion.

From the practical standpoint it is interesting to note that Stoklasa (**183**) finds that different plants have different selective abilities towards cations and anions, respectively. The Gramineae, in his experiments, absorb anions more than cations; potatoes and beets more cations than anions; legumes anions and cations equally. Of course, further confirmation of such results is necessary.

2. Ion antagonism

There is sufficient evidence to show that Ca and Mg, which precipitate sols, cause contraction and impermeability and that K and Na, which stabilize sols, cause relaxation on the one hand, and greater permeability on the other. The antagonistic effects between univalent and divalent cations in colloidal systems have been studied by Meulen and Rieman (**113, 159**), by Weiser (**203**), and also by Höber (**71**). Simms (**173**) has derived a mathematical expression for the antagonism between Na (or K) and Mg (or Ca) in true (non-colloidal) solutions of oxalate One might expect—although it has not been demonstrated—that the activity of proteins would show a similar antagonism.

It is essential, however, for the investigators to keep in mind the fact that the effect of a mixture of two or more cations (or anions) on cell permeability may be quite different from the effect of one alone when the activity of the ions is due to different combining proportions of the cations (or anions) in a mixture (such as the soil solution), in proportion to the relative combinations in solutions of each alone (**174**). Some of the practical agricultural applications of ion antagonism between Ca and K have been investigated by Lipman *et al.* (**98**), by McCool and Weldon (**106**) and by Fonder (**42**).

3. Gibbs-Donnan distribution law

In systems containing a non-diffusible ion, a marked effect is observed on the unequal distribution of inorganic electrolytes on either side of the cell membrane. A non-diffusible electro-negative ion will produce a greater concentration of diffusible electro-positive ions and a lower concentration of electro-negative ions on its own side of the membrane than on the other. A non-diffusible electro-positive ion will produce the opposite effect. At equilibrium the product of the concentrations of each pair of oppositely charged diffusible ions is the same on either side (**35, 36**). The distribution of an electrolyte in such a system will be expressed by the equation:

$$\frac{x_i\, y_i}{v_i^{\,2}} = \frac{x_e\, y_e}{v_e^{\,2}} \qquad (12)$$

where x_i and y_i represent the amounts of cation and anion, respectively, within the membrane at equilibrium; and x_e and y_e the equilibrium amounts of the same pairs of ions in the external solution; v_i and v_e the volume of the solvent on either side of the membrane.

The importance that the Gibbs-Donnan distribution law may have in the equilibrium conditions and consequent selectivity of nutrients by plants has been mentioned by Hoagland (**67**), Pierre and Parker (**148**), and also by Briggs and Petrie (**14**). It will be recalled (**187**) that Parker (**141**) found that the displaced soil solution from many of the experimental fields in America were too low in inorganic phosphorus to support growth in the nutrient media when the phosphorus in these soil solutions was used as the source of phosphorus. One of the explanations offered by him to explain this anomaly is that a Donnan membrane equilibrium may exist, resulting in a higher concentration of phosphorus in the solution on the surface of the soil particles than in the soil solution itself. The mechanism involved has already been discussed by the writer (**132, 187**). But the exact experimental verification of Donnan's law in the case of specific plant tissue is difficult, for it is impracticable to measure the x_i, y_i, and v_i accurately, since a calculation of the amount of ions absorbed by means of the difference between their concentration before and after equilibrium is reached does not take cognizance of the amounts of these ions already present in the tissue (**14**). Hence, extension of the methods of Osterhout (**135**) and of Osterhout and Dorcas (**137**) on the absorption of CO_2 by *Valonia* do not afford a basis for generalization, for even here the average concentration of the ions in the cell, as a whole, is not considered but only that in the cell sap.

As BRIGGS and PETRIE (**14**) point out, in the plant cell we are dealing with a polyphase system. The DONNAN equilibrium applies to free ions only. In the cell there is evidence that some of the ions are present as undissociated salts of proteins or other substances, or again some of the ions may be physically absorbed. We should expect, therefore, that the simple DONNAN equation would not hold in such systems. That this is the case is apparent from the results of BRIGGS and PETRIE's experiments on the absorption of 0.1 M KCl and NH_4Cl from carrot tissues (**14**) and from WRIGHT's (**214**) work on the secretion of high amounts of calcium in milk from the relatively low concentration of this element in the blood plasma. The latter finds that both the degree of dissociation of the protein salts and the establishment of a DONNAN equilibrium must be taken into account in explaining the inequalities of the products in the distribution of inorganic elements in living tissues, whereas the former (**14**) have deduced that the apparent ionic internal product, resulting from the collective effect of all the phases within the tissue, must have a higher value than that of the external media. BRIGGS and PETRIE (**14**) point out that "the plant cell is not a simple system, composed of a mere membrane enclosing a homogeneous solution, one of the ions of which is indiffusible; there are at least three phases between which the ions may be distributed—the external medium, the cytoplasm, and the vacuoles. Within the cytoplasm also, and perhaps in the cell-sap, there are micelles, or gel-particles, which can constitute yet other phases."

BUTKEWITSCH and BUTKEWITSCH (**17, 18**) have advanced as a reason for the inequality of the products of the diffusible ions on each side of a membrane in systems containing a non-diffusible ion, that the degree of permeability of the plasma membrane is not the same in both directions. This has been shown to be the case by WERTHEIMER (**204, 205**) for certain types of membranes. In such a case the equation of membrane equilibrium will contain, besides the concentration product of diffusible ions, a factor corresponding to the velocity of migration of the corresponding ionic pairs. Thus, if a solution, say of KCl, is separated by a membrane permeable in the two directions, we have:—

$$\begin{array}{cc|cc} x' & K^+ & K^+ & x'' \\ y' & Cl^- & Cl^- & y'' \\ & p' \;— & \rightarrow & \\ & \leftarrow & — \; p'' & \end{array}$$

where $x'\,y'\,p' = x''\,y''\,p''$

where x and y represent the concentration of K and Cl ions and p a permeability factor.

By this means it can be calculated (**17**) that in OSTERHOUT's experiments on *Valonia* (**134**) the permeability of the plasma membrane for Na in one direction is one-fifth that in the opposite direction and in the case of K the difference is 45 times.

Within the limitations already discussed it is possible to interpret the experiments of BUTKEWITSCH and BUTKEWITSCH (**17**) on the influence of colloidal silicic acid upon the absorption of phosphorus by maize plants by DONNAN's principle. When small amounts of P_2O_5 are present in the nutrient medium, growth and absolute amounts of P_2O_5 increased by the additions of silicic acid in the nutrient medium; but the *percentage* of P_2O_5 remained about the same. It follows that a functional replacement of P_2O_5 by H_2SiO_3 in the plant does not occur, but that the favorable action of H_2SiO_3 on the development of the plants is due to its effect in increasing the absorption of P_2O_5 in a medium that contained minimum amounts of phosphorus for growth (**189**).

4. THE EFFECT OF DIFFERENCES IN POTENTIAL BETWEEN SOIL AND PLANT

Since the breaking down of carbohydrates sets free electromotive force, part of the energy stored in the plant must be electrical and used in growth. The work of GROH (**49**) is important. He attributes the different behavior of different plants toward different degrees of hydrogen-ion concentration in the medium to a difference in potential between the plant and soil solutions, which results in a decomposition of the nutrient material. This hypothesis is supported by the following experiment. Peas and also barley were grown in sand and also in soil cultures to which $Ca(NO_3)_2$ were added. During growth individual plants were connected with a galvanometer with one of the platinum electrodes immersed in the soil and the other connected to the plant. The E.M.F. produced varied with conditions—from 0.01 to 0.20 volts. The greater the distance from the roots, of the electrode connected to the plant, the greater the current registered. This shows that the difference of potential existing in the plant itself is much less than that existing between the plant and the soil. The degree of vegetative development apparently was not a large factor in determining the degree of the difference of potentials noted. GROH's work, although of a preliminary nature, is suggestive and should be considered in connection with OSTERHOUT's (**136**) more refined work on the potential difference in *Valonia* and *Nitella*.

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THE DETERMINATION OF NONPROTEIN NITROGEN IN ONE-TENTH OF A CUBIC CENTIMETER OF BLOOD*†

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THE development of accurate methods for the determination of important blood constituents in small quantities of blood should prove of value to those working with small animals, such as rats, where only small quantities of blood are available. Such methods also should be welcomed in the clinical laboratory, especially in those cases where it is difficult to obtain blood from a vein.

There are several references in the literature to micromethods for the determination of nonprotein nitrogen in 0.1 and 0.2 c.c. of blood. Among these we mention those of Peters,[4] Wilhelmj,[7] Ponder,[5] Kleinmann,[3] and Taylor and Hulton.[6] Wilhelmj, and Taylor and Hulton have compared the nonprotein nitrogen content of venous and capillary blood and have found it to be the same.

This paper reports a modification of the Folin-Wu[2] method for the determination of nonprotein nitrogen which requires only 0.1 c.c. of blood. In this modification the sodium tungstate and sulphuric acid are mixed and diluted to form one precipitating agent. The precipitated protein is separated by centrifuging and the final digestion is made in a test tube graduated at 5 c.c.

PREPARATION OF REAGENTS

1. Precipitating Mixture: To 35 c.c. of water add 5 c.c. of 10 per cent sodium tungstate solution and 5 c.c. of 2/3 N. sulphuric acid.

2. Acid Digestion Mixture: To 405 c.c. of water add 5 c.c. of 5 per cent copper sulphate solution, 30 c.c. of 85 per cent phosphoric acid and 10 c.c. of concentrated sulphuric acid. This solution is Folin and Wu's dilute acid digestion mixture diluted 1 to 5.

3. Standard Ammonium Sulphate Solution: Dissolve 0.4716 gm. of pyridine base free ammonium sulphate in water and dilute to a volume of 1 liter.

4. Nessler's Solution: Prepared according to the directions of Folin and Wu.[2]

PROCEDURE

Sterilize a finger tip with alcohol and after allowing it to dry thoroughly, puncture it with a lancet and collect 3 drops of blood on a small watch glass. By means of a Folin[1] capillary pipette, transfer 0.1 c.c. of blood from the

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watch glass to 1.9 c.c. of freshly prepared precipitating mixture in a centrifuge tube, keeping the tip of the pipette beneath the surface of the liquid. Since this pipette is made to hold 0.1 c.c., the blood is washed out by drawing the liquid up and down in the pipette. Finally, the mixture is thoroughly stirred, using the pipette as a stirring rod. In measuring, care should be taken to remove any blood from the tip of the pipette by wiping it with a filter paper or clean cloth. The mixture is allowed to stand for twenty-five minutes and then centrifuged rapidly for five minutes. We have obtained low values if the mixture has not stood for twenty-five minutes before centrifuging.

After centrifuging, transfer 1 c.c. of the clear supernatant liquid to a dry Pyrex digestion tube (13 × 125 m.m.) graduated at 5 c.c. and add 0.5 c.c. of the acid digestion mixture. Digest carefully over a microburner, with a continuous shaking. When the solution turns brown, continue the heating over a low flame until the solution becomes a pale green in color. Cool for one minute, add 3 c.c. of water and shake until a uniform solution is obtained. Next add 1.5 c.c. of Nessler's solution, make up to volume with water and mix thoroughly. Compare this in a colorimeter with a standard prepared by placing 3 c.c. of standard ammonium sulphate solution, 10 c.c. of acid digestion mixture, and 50 c.c. of water in a 100 c.c. volumetric flask and mixing thoroughly. Add to this 30 c.c. of Nessler's solution, make up to volume with water and mix thoroughly. The following formula may be used for calculating results:

$$\frac{\text{Reading of standard} \times 30}{\text{Reading of unknown}} = \text{mg. of nonprotein nitrogen per 100 c.c. of blood.}$$

In the above method the proportions of reagents used are the same as in the Folin and Wu procedure, except the precipitating mixture. Keeping this the same would give a final volume of 1 c.c. in the centrifuge tube. This was tried but it was very difficult to separate 0.5 c.c. of clear liquid after centrifuging. By making the final volume 2 c.c., 1 c.c. of clear liquid may be removed easily. Although twice the volume of precipitating mixture is used, it is not diluted, because this concentration is necessary for the complete precipitation of blood proteins. We have obtained high values in using a dilute precipitating mixture.

The acid digestion mixture of Folin and Wu has been diluted 1-5 to facilitate measuring. It was felt that 0.5 c.c. could be measured more accurately than 0.1 c.c.

It is obvious that the method described requires careful technic and accurately calibrated apparatus. One source of error has been the ammonia in the laboratory fumes. Small traces of ammonium salts on centrifuge and digestion tubes may lead to large errors. One must make sure that all apparatus is clean and ammonia-free before using. For color comparisons, a microcolorimeter has proved very satisfactory because of the small amount of sample available; however, any colorimeter with narrow cups is satisfactory.

Table I shows the results obtained with the new micromethod as compared with the Folin and Wu method; it also shows a comparison of values

TABLE I

A COMPARISON OF RESULTS OBTAINED BY THE FOLIN-WU METHOD AND THE NEW MICROMETHOD FOR THE DETERMINATION OF NONPROTEIN NITROGEN ON VENOUS BLOOD; ALSO A COMPARISON OF THE COMPOSITION OF VENOUS AND CAPILLARY BLOOD AS DETERMINED BY THE NEW MICROMETHOD

BLOOD SAMPLE	VENOUS BLOOD		CAPILLARY BLOOD
	FOLIN-WU	NEW MICROMETHOD	
	MG. NONPROTEIN NITROGEN PER 100 C.C. OF BLOOD		
H. W.	35.2 34.9 34.9 Ave. = 35.0	35.1 34.3 35.1 Ave. = 34.8	34.1 35.2 35.2 Ave. = 34.8
O. H.	32.6 32.6 32.6 Ave. = 32.6	32.4 31.1 32.0 Ave. = 31.8	32.4 32.4 30.8 Ave. = 31.5
L. S.	38.2 38.0 38.0 Ave. = 38.1	37.5 37.5 37.5 Ave. = 37.5	38.0 37.0 37.5 Ave. = 37.5
L. L.	33.0 33.3 33.0 Ave. = 33.1	33.3 32.4 34.3 Ave. = 33.3	33.9 33.2 33.2 Ave. = 33.4
C. V.	37.3 36.8 36.8 Ave. = 37.0	35.1 36.4 Ave. = 35.7	37.0 36.4 36.6 Ave. = 36.7
S. B.	29.7 29.3 29.3 Ave. = 29.4	31.3 29.3 29.3 Ave. = 30.0	32.6 32.3 29.7 Ave. = 31.4
P. A.	34.9 35.7 35.5 Ave. = 35.4	35.5 35.2 34.1 Ave. = 34.9	34.5 35.2 33.3 Ave. = 34.3
J. L.	29.6 29.7 30.0 Ave. = 29.8	30.2 29.6 29.7 Ave. = 29.8.	30.0 29.6 29.7 Ave. = 29.8

obtained on venous and capillary blood. The results of the two methods agree closely. It is also evident that capillary blood has the same nonprotein nitrogen content as venous blood.

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